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Accommodation in Myelinated Nerve Fibres of Xenopus Laevis as Computed on the Basis of Voltage Clamp Data

By

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Abstract

Frankenhaeuser B and Å. B Vallbo *Accommodation in myelinated nerve fibres of Xenopus laevis as computed on the basis of voltage clamp data* Acta physiol scand 1965 63 1—20 — The equation system earlier derived from the voltage clamp analysis on myelinated nerve fibres from *Xenopus laevis* was programmed for a digital computer The response of the nerve model to linearly rising currents was computed for a number of quantitative modifications of the nerve model The agreement between the computations and the earlier experimental results was qualitatively satisfactory The quantitative agreement was not perfect but the discrepancies were not greater than what is expected from the known errors in the voltage clamp technique The computations indicated that changes of any of the constants of the nerve model have effects on the rate of accommodation The greatest effects were obtained by changing the inactivation of the sodium permeability It was concluded on the basis of the results obtained in the present and in the earlier investigation, that the slow excitability changes of the nerve fibres are well predicted by the equation system describing the ionic currents Further it was concluded that the normal variation in accommodation among myelinated nerve fibres from *Xenopus laevis* is accounted for mainly by variations in the rate constants $_h$ although this may not be the only variable of importance A variation in the turning on of the potassium permeability might also be significant

One of the excitability properties of nerve is that the nerve accommodates to applied currents Accommodation has been measured in various ways *e g* by sinusoidal currents by smoothly increasing currents or by conditioning and test currents (see Katz 1939) Accommodation varies not only among nerves from different species of animals (*e g* Solandt 1936) but also among fibres within the same nerve (*e g* Tasaki 1950 Sato 1951 Vallbo 1964 a) The ionic theory for impulse generation ought to account for accommodation (Hodgkin 1963) The voltage clamp analysis has given the ionic theory a quantitative form sufficiently complete to allow a further treatment of the problem of accommoda

tion The squid fibre analysis (Hodgkin and Huxley 1952 a—d, Hodgkin, Huxley and Katz 1952) has been carried far enough to indicate the general behaviour of the membrane, but not far enough to account for variations in accommodation Calcium has an effect on accommodation (*e g* Katz 1936) and the influence of calcium on the squid nerve has been analysed in detail (Frankenhaeuser and Hodgkin 1957, Huxley 1959) The major bulk of the voltage clamp data for the myelinated nerve fibre has been lumped together with the aim to cover the general properties of the myelinated nerve fibre (Dodge and Frankenhaeuser 1959, Frankenhaeuser 1959 1960, 1962 a b, c, 1963 a b) An inspection of the empirical equations, which describe the ionic currents, reveals that a change of almost every constant the rate constants as well as the permeability constants, will change the accommodation To judge how much a single change of a constant will affect accommodation is a too complicated task to be fruitful without a complete numerical computation

Voltage clamp experiments together with excitability measurements have been performed on single myelinated nerve fibres in order to elucidate the problem of accommodation in further detail (Vallbo 1964 *b*) These experiments have revealed that a variation of the inactivation rate constant α_h is significantly correlated to the variation in accommodation among the fibres The experimental study could not decide how far the variation in α_h is sufficient to account quantitatively for the variation in accommodation, because variations in other constants appeared too

Numerical computations seem to be the best method at this stage to decide by which factors the rate of accommodation is affected and how much it is affected by the various factors In the present investigation computations were made of the membrane response to linearly increasing stimulating currents and the critical slope for excitation of the quantitative nerve model was computed for a number of quantitative modifications of the model The modifications were chosen so that they reasonably cover the variations earlier found in experimental investigations

It will be shown that the response of the nerve model to linearly increasing currents is in reasonable agreement with the experimental excitability measurements Deviations were noted but these were not larger than those expected on the basis of the various uncertainties in the experimental analysis Changes of the sodium carrying mechanism as well as changes of the potassium carrying mechanism had a clear effect on the critical slope

Methods

The nomenclature the symbols and the equation system to be computed are the same as those given by Frankenhaeuser and Huxley (1964) The computations were made with the digital computer Wegematic (Computor Division Karolinska Institutet Stockholm) The integration was made with the Runge Kutta fourth order method (*e g* Hamming 1962)

Computations were made of the response of the nerve model to linearly increasing currents. The slope of the current was changed inbetween the runs in such a way that successive slopes rapidly converged towards that which was critical for excitation. The current threshold for a step current of one msec duration was also determined within $\pm 0.5\%$. This threshold current was used as an approximation for the rheobasic current. In order to facilitate comparison with the experimental results a threshold potential (V_t) was also determined. Threshold potential (V_t) is here defined as the potential at which the rate of change of membrane potential was 80 volts/sec during regenerative activity when a nearly threshold step current pulse was applied.

Modifications of the 'standard data list'. The 'standard data list' as given by Frankenhaeuser and Huxley (1964), which applies for a temperature of 20° C, was used as the basis of the present investigation with the exception that the constant B in the equation for the rate constant α_h (Frankenhaeuser and Huxley 1964, eqn. 11) was taken, in most of the computations, as -22 mV instead of -10 mV. The nerve model according to the 'standard data list' had a lower critical slope than those seen in myelinated nerve fibres from *Xenopus laevis* and the computations were very time consuming. This change of the rate constant α_h had the effect that the critical slope of the nerve model better agreed with that of the nerve fibres and it decreased the computation time considerably. The 'standard data list' with this modification was therefore used in the majority of the computations. Simple modifications of the data list were made. The modified data list will be indicated below by the modifications only, implying that the other values are given by the 'standard data list'.

The constants of the 'standard data list' were varied in such a way that the variations in the nerve model would reasonably cover the variations experimentally found among myelinated nerve fibres (Frankenhaeuser 1960, 1963 a, Vallbo 1964 b). One exception was made from this principle. Computations were not made with higher values for the rate constant α_h at corresponding potentials than those given by the 'standard data list'.

The modifications of the rate constants were made by changing only B in the equations for the rate constants (Frankenhaeuser and Huxley 1964, eqns. 11—14) and by an amount which was roughly equivalent to the net variation of the rate constants found experimentally in the range of membrane potential (V) between 20 and 30 mV. The experimentally found variations in A and C were thus compensated for by the variation in B. A suffix indicates which rate constant is concerned, thus e.g. $B_{\alpha h}$ for the constant B in the equation for the rate constant α_h. The modifications made and the reasons why just these modifications were made will be summarized here.

(1) The inactivation of the sodium carrying mechanism (h) was varied by changing the rate constant α_h only, since it has been shown that the main variation in the inactivation among nerve fibres is due to variations in the rate constant α_h (Frankenhaeuser 1960, Vallbo 1964 b). The constant $B_{\alpha h}$ (Frankenhaeuser and Huxley 1964, eqn. 11) was varied. This shifted the $\alpha_h - V$ curve along the voltage axis without causing changes in the shape of the curve. The shape of the $h_\infty - V$ curve was only little affected by these changes of $B_{\alpha h}$. It seemed therefore justified to consider the potential at which the inactivation of the sodium permeability is half complete in the steady state (V_h) for comparisons between the effect of changes in the inactivation and the effect of changes in the potential dependence of the potassium mechanism and also for comparing the computed solutions with the results obtained in the experimental study (Vallbo 1964 b).

An equation of the same form as the one used by Frankenhaeuser (1959, eqn. 1) was fitted to the computed values of the steady state inactivation. The constant k_h was 4.5 mV in the equation which gave the best fit. Thus the $h_\infty - V$ curve of the 'standard data' was steeper than that of the nerve fibres investigated which had a value for k_h

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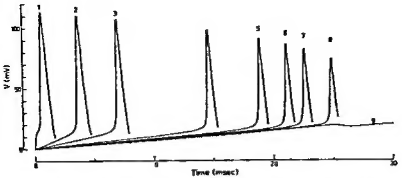

Fig 1 Computed membrane potential changes in response to step current stimulation (1) and linearly rising currents (2)—(9) Stimulating current was in (1) 0 438 mA/cm or 1.20 rheobases. Slopes of linearly rising stimulating currents were in (2) 125 00 mA/cm ,sec or 343.27 rheobases/sec (3) 62 50 mA/cm /sec or 171 63 rheobases/sec (4) 31.25 mA/cm ,sec or 85 82 rheobases/sec (5) 25 39 mA/cm ,sec or 69 73 rheobases/sec (6) 23 44 mA/cm sec or 64.36 rheobases sec, (7) 22 46 mA/cm /sec or 61 68 rheobases/sec (8) 21 48 mA/cm /sec or 59 00 rheobases sec and (9) 21.24 mA/cm /sec or 58 33 rheobases/sec. Standard data except $E_{a_k} = -16$ mV

1964 b) A minute further decrease of the slope changed the response of the membrane from a clear regenerative action potential into a subthreshold response and a delayed rectification The threshold for excitation did not change much with these changes in the slope of the stimulating current The threshold was estimated as the density of the stimulating current (threshold current) or as the membrane potential (threshold potential) in either case at the foot of the action potential when the rate of change of membrane potential was 80 volts/sec The density of the stimulating current at this moment was 0 431 mA/cm² when the slope of the stimulating current was 125 mA/cm²/sec (trace 2 in Fig 1) and 0 529 mA/cm² when the slope was 21 48 mA/cm²/sec (trace 8) The latter slope was the minimum slope for a regenerative action potential Thus the threshold current measured in this way increased by only 22 7 % when the slope was changed from a rather steep one to the critical slope for excitation The threshold potential was 21 10 mV and 28 15 mV respectively in these two computations These findings indicated that accommodation as measured here does not imply a marked increase in threshold for an action potential (*cf* Tasaki 1950 Frankenhaeuser 1952 Vallbo 1964 b)

Fig 1 shows the type of response which was most commonly seen when linearly rising currents were applied However another type occurred when the curve for the rate constant α_k was sufficiently much shifted along the voltage axis to the right This response which appeared only when the slope of the linearly rising current was near the critical slope consisted of oscillations in the membrane potentials in the currents in the permeability variables and in the permeabilities Small oscillations started at a membrane potential of about 17 mV They increased in size with time and assumed the shape and size of

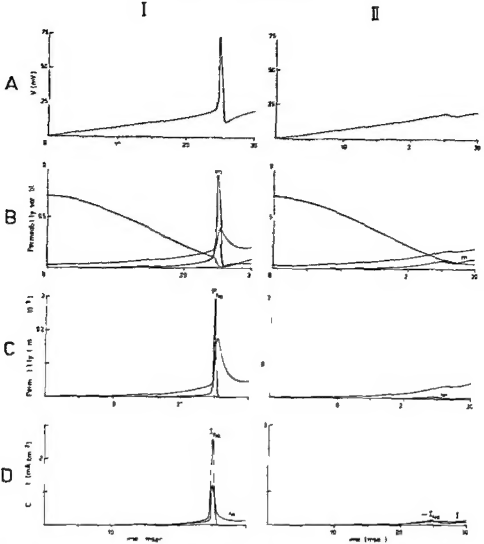

Fig. 2. Computed membrane potential changes (A), permeability variables h, m and n (B), sodium and potassium permeabilities (C), and sodium and potassium currents (D) in response to linearly rising stimulating currents. (I) Slope of stimulating current 21.48 mA/cm²/sec which is 59.00 rheobases/sec. (II) Slope of stimulating current 21.24 mA/cm²/sec which is 58.33 rheobases/sec. Standard data except $E_{Qk} = -16$ mV. Same computations as illustrated in Fig. 1, trace 8 and trace 9.

small action potentials at higher slopes of the stimulating current. At lower slopes the oscillations remained small and gradually faded away. There seemed to be a continuous increase in the amplitude of the membrane responses from small oscillations into clear action potentials as the slope of the current was increased. It was therefore not possible to give a definite minimum slope for a

Table I Currents permeability variables and permeabilities in the resting state and at $V = 16$ mV in the steady state and during stimulation by linearly increasing currents near the critical slope for excitation from the computations illustrated in Fig 2 I and II Standard data except $B_{\alpha_h} = -16$ mV

	Units	Resting value	Steady state value	Fig 2 I at 21 70 msec	Fig 2 II at 22 04 msec
V	mV	0	16 0	16.0	16 0
I	mA/cm^2	*0*		*0 466*	*0 467*
dV/dt	volts/sec	0	0	1 0000	0.9500
h		0 7073	0 0620	0 1720	0 1695
m		0 0005	0.0436	0 0420	0 0425
n		0.0768	0 1548	0 1290	0 1295
p		0.0049	0 0757	0 0222	0 0223
P_{Na}	cm/sec × 10^{-3}	0 0000	0 0009	0 0075	0 0074
P_K	cm/sec × 10^{-3}	0 0009	0.0788	0 0199	0.0201
P_p	cm/sec × 10^{-3}	0 0000	0 0004	0.0003	0.0003
I_{Na}	mA/cm^2	−0 000	−0 025	−0 066	−0 065
I_K	mA/cm^2	0 001	0 077	0 053	0 054
I_p	mA/cm^2	*−0 000*	*−0 010*	*−0.007*	*−0.007*
I_L	mA/cm^2	−0 001	0 484	0 484	0 484

regenerative action potential The critical slope of the nerve model is given as the range between the minimum computed slope that gave rise to a clear action potential and the computed maximum slope that did not elicit an action potential This range was as a rule 0 97 mA/cm^2/sec which corresponds to about 2 5 rheobases/sec A somewhat wider range is given for the computations in which oscillations occurred since then there was no sharply defined critical slope

Membrane potential permeability variables permeabilities and ionic currents during linearly rising currents

Fig 2 shows the computed membrane potential (A) the permeability variables m h and n (B) the sodium and potassium permeabilities (C) and the sodium and potassium currents (D) during two linearly rising currents of only slightly different slopes The difference in the slope of the stimulating current between the computations illustrated in I and II was 0 24 mA/cm^2/sec or 0 67 rheobases/sec It is seen that at times less than about 22 msec, the potential changes the permeability variables the permeabilities and the ionic currents differed only negligibly between the two runs At longer times however th
was a marked difference as the membrane fired an

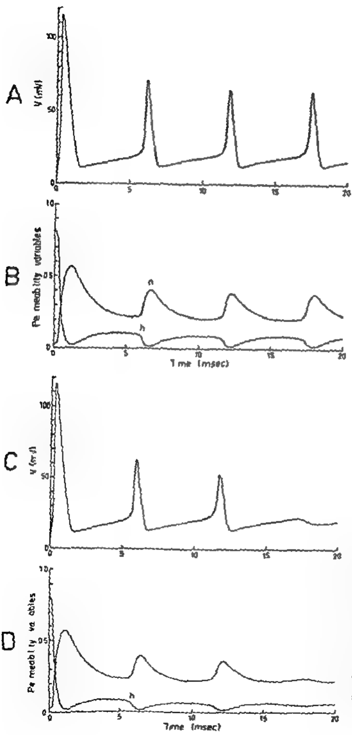

Fig. 3. Computed membrane potential changes (A and C) and permeability variables h and n (B and D) in response to applied step currents of long duration. (A and B) standard data (B_{α_h} = −10 mV), stimulating current 0.6 mA/cm. (C and D) standard data except B_{α_h} = −11 mV, stimulating current 0.65 mA/cm.

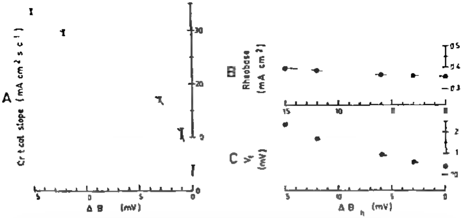

Fig 4 Effect on critical slope rheobase and threshold potential (V_t) of changes of the potential dependence of the inactivation of the sodium permeability Standard data except variations of $B_{\alpha h}$ from the standard value (− 10 mV) Abscissae changes of $B_{\alpha h}$ Ordinates (A) critical slopes in units of mA/cm²/sec (B) rheobase in mA/cm² (C) threshold potential (V_t) in mV The symbols in (A) give the range between the computed minimum slope that gave rise to an action potential and the computed maximum slope that did not elicit an action potential At $B_{\alpha h}$ = − 10 mV (standard value) only an upper limit of this range is given The extreme values of the critical slope in units of rheobases/sec were 86 24 and less than 13 70 rheobases/sec respectively

not in II Thus a very small change of the conditions may change the response of the membrane radically Table I gives a number of values from the computations illustrated in Fig 2 I and II at V = 16 mV and also the steady state values at this potential and the corresponding resting values The potential V = 16 mV was chosen because it was very close to the foot of the action potential in I The variable m was very close to its steady state value at this potential while n and especially h had not approached as closely to their steady state values It is clear that values corresponding to those given in Table I varied somewhat from one computation to the other depending upon which modifications of the standard data list were made for that particular computation The maximum values of the permeabilities and the ionic currents during the action potential were considerably higher in response to step current stimulation than in response to stimulation by linearly rising currents of a critical slope for excitation The sodium and potassium permeabilities reached peak values which were about 10 and 50 per cent respectively of those attained in response to step current stimulation The peak values of the sodium and potassium currents were about 50 and 35 per cent respectively of the peak values on step current stimulations This comparison was made between the computations illustrated in Fig 1 trace 1 and that illustrated in Fig 1 trace 8 and Fig 2 I Fig 2 I D shows further that the sodium current was single peaked when a stimulating current of critical slope was applied while the sodium current has two maxima on step current stimulations (Frankenhaeuser and Huxley 1964)

Fig. 5 Effect on critical slope of changes of the inactivation of the sodium permeability Standard data except variations in B_{a_h} from the standard value (— 10 mV) Abscissa changes of potential at which the inactivation is half complete in the steady state (V_h) from its standard value (7 3 mV) Ordinate critical slope in units of mA/cm /sec Same computations as illustrated in Fig 4 A

Repetitive discharges

Repetitive firing in response to stimulation by step currents of long duration occurred in the standard fibre (Fig 3 A and B) It seemed that the repetitive firing would go on for infinite time since the same periods were repeated after the first 20 msec as long as the computation was continued The inactivation (h) of the sodium permeability did not reach values larger than 0 1 after this time Computations of the response to step currents of long duration were also made when the data were modified by changing B_{a_h} from — 10 mV to — 11 and — 13 mV Repetitive firing could not be elicited when B_{a_h} was — 13 mV A few action potentials (maximum seven) occurred when B_{a_h} was — 11 mV in response to step current stimulation (Fig 3 C and D)

Effects on critical slope of variations in the constants

The critical slopes were affected by changes in a number of constants as shown in Fig 4 to 10 The effect of changes in the inactivation of the sodium carrying mechanism is shown in Fig 4 A The critical slope is here plotted against the change in B_{a_h} from its standard value of — 10 mV The constant B_{a_h} was changed by as much as 15 mV from its standard value This shifted the position of the h_∞ — V curve along the voltage axis by 8 3 mV The experimental analysis of the nerve fibres showed that the position of the h_∞ — V curve along the voltage axis varied by about 10 mV relative to threshold potential and that this variation was mainly accounted for by a variation in the rate constant a_h It is evident that the critical slope depended on the rate constant a_h but that this dependence was rather complex The results from Fig 4 A are also shown in Fig 5 in which the critical slope is given as a function of the potential at which the inactivation is half complete in the steady state

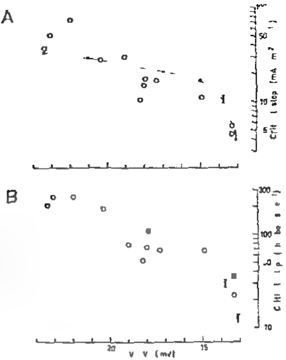

Fig 6 Comparison between recorded and computed critical slopes Abscissae potential difference $V_h - V_r$ in mV Ordinates (A) critical slope in units of mA/cm²/sec (B) critical slope in units of rheobases/sec Open circles experimental results (Vallbo 1964 b) Bars computed critical slopes from the computations illustrated in Fig 4 and 5

(V_h) In order to compare these findings with the experimental results (Vallbo 1964 b) the computed critical slopes were plotted against the potential difference $V_h - V_r$ (Fig 6) and the previous experimental findings were plotted on the same graphs without any form of arbitrary scaling The ordinates are logarithmic in order to compress the scales As was pointed out earlier the $h_\infty - V$ curve was steeper in the nerve model than in the nerve fibres It might therefore not be appropriate to stress the agreement between the absolute values from the two sets of results The graphs do however show partly how great an effect variations in inactivation have on the critical slope of the nerve model partly the relation between the variations in the inactivation and the variations in the critical slope actually found among myelinated nerve fibres The general agreement between the two sets of results from this point of view seems rather striking considering that other factors which also affected accommodation varied from one fibre to another

The critical slopes as affected by changes of the membrane potential dependence of the potassium-carrying system is shown in Fig 7 A The modifications were made by changing B_α and $B\beta_n$ simultaneously The maximum shift of the rate constants was 12 mV in these computations It is difficult to estimate the variation of the membrane potential dependence of the potassium permeability among nerve fibres in the significant potential region (Franken

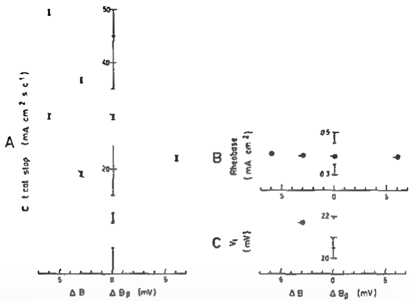

Fig 7 Effect on critical slope rheobase and threshold potential (V_t) of changes in the potential dependence of the potassium permeability Standard data except $B_{a_h} = -22$ mV (upper curve in A) and -11 mV (lower curve in A) and variations in B_α and B_β by the same amount from their standard values ($B_{\alpha_n} = 35$ mV $B_\beta = 10$ mV) Abscissae changes of B_{α_n} and B_β Ordinates (A) critical slope in units of mA/cm /sec (B) rheobase in mA/cm ($B_{a_h} = -22$ mV) (C) threshold potential (V_t) in mV ($B_{a_h} = -22$ mV) The extreme values of the critical slope in units of rheobases/sec were 126 36 and 58 73 rheobases/sec respectively when B_{a_h} was -22 mV

haeuser 1963 a) because the resolution of the voltage clamp technique is limited in this range but it seems likely that the variations among the nerve fibres are of the same order of magnitude as the changes used in these computations Computations were made with this type of modification at two different values of B_{a_h} This constant was -22 mV in the computations illustrated by the upper curve in Fig 7 A and -11 mV in the computations illustrated by the lower curve The effect on the critical slope of the changes in the turning on of the potassium permeability was considerable and it was different at the two values of the constant B_{a_h}

The effect on the critical slope of variations in the inactivation of the sodium permeability and the effect of corresponding variations in the turning on of the potassium permeability were of the same order of size over a large range but the inactivation had a considerably greater effect when a_h was sufficiently much shifted to the right (Fig 5 and Fig 7 A)

The sodium and potassium permeability constants ($\bar{P}_{Na}$ and P_K) were varied in the computations between 4 0 and 12 0 and between 0 6 and 1 4 × 10^{-3} cm/sec respectively The means and the standard deviations of these permeability constants measured on nerve fibres are 8 3 ± 3 6 and 0 93 ± 0 19 × 10^{-3} cm/sec

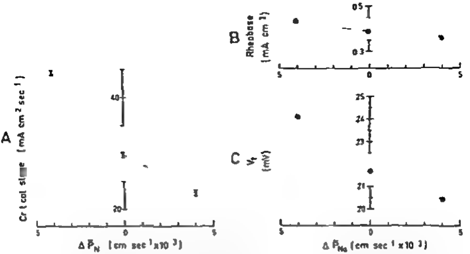

Fig 8 Effect on critical slope rheobase and threshold potential (V_t) of changes in the sodium permeability constant ($\bar{P}_{Na}$) Standard data except $B_{\alpha_h} = -29$ mV and variations in the sodium permeability constant from its standard value ($8\,0 \times 10^{-}$ cm/sec) Abscissae changes of $\bar{P}_{Na}$ Ordinates (A) critical slope in units of mA/cm²/sec (B) rheobase in mA/cm² (C) threshold potential (V_t) in mV The extreme values of the critical slope in units of rheobases/sec were 103 85 and [illegible] 56 rheobases/sec respectively

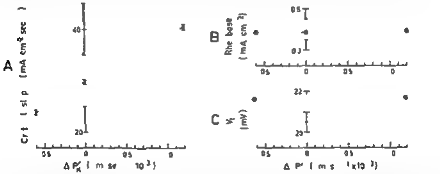

Fig 9 Effect on critical slope rheobase and threshold potential (V_t) of changes in the potassium permeability constant (P_K) Standard data except $B_{\alpha_h} = -29$ mV and variation in the potassium permeability constant from its standard value ($1\,2 \times 10^{-}$ cm sec) Abscissae changes of P_K Ordinates (A) critical slope in units of mA/cm²/sec (B) rheobase in mA/cm² (C) threshold potential (V_t) in mV The extreme values of the critical slope in units of rheobases/sec were 104 83 and 64 07 rheobases/sec respectively

(Vallbo 1964 b) The changes of $\bar{P}_{Na}$ (Fig 8) and P_K (Fig 9) had a moderate effect on the computed critical slope It is clear that the effects could be some what different at other values of other constants in the nerve model than those used here *e g* at other values of B_{α_h}

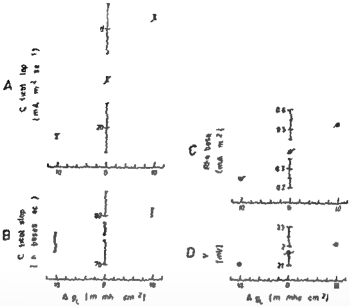

Fig. 10. Effect on critical slope, rheobase and threshold potential (V_t) of changes in the leak conductance (g_L). Standard data except θ_{0h} = − 22 mV and variation in the leak conductance from its standard value (30.3 m mho/cm²). Abscissae: changes of g_L. Ordinates: (A) critical slope in units of mA/cm²/sec; (B) critical slope in units of rheobases/sec; (C) rheobase in mA/cm²; (D) threshold potential (V_t) in mV.

The leak conductance (g_L) was varied from 20.0 to 40.0 m mho/cm² in the computations. The mean of the leak conductances measured on nerve fibres is 30.3 m mho/cm² (Frankenhaeuser 1960) and 20.9 ± 7.0 m mho/cm² (mean and S.D.) (Vallbo 1964 b). The changes of g_L (Fig. 10) affected the critical slope moderately as measured in units of mA/cm²/sec but the effect was considerably less on the critical slope as measured in units of rheobases/sec.

Effects on rheobase and threshold potential of changes in the constants

The effects on the rheobase and the threshold potential (V_t) of changes in the various constants are shown in most of the above graphs and in Fig. 11. These thresholds, as measured by a step current of one msec duration, were not much affected by most of the changes. The exceptions were that a shift of the rate constants α_m and β_m was associated with a marked change of the rheobasic current and the threshold potential. The latter changed by about the same amount as the shift of the two constants along the potential axis (Fig. 11). A change of g_L had a marked effect on the rheobasic current, about proportional to the change in g_L, but the effect on the threshold potential was negligible (Fig. 10).

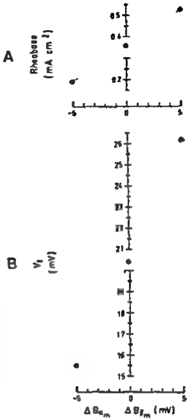

Fig 11 Effect on rheobase and threshold potential (V_t) of changes in the turning on of the sodium permeability (m). Standard data except simultaneous variations of B_{α_m} and B_{β_m} by the same amount from their standard values ($B_{\alpha_m} = -2?$ mV $B_{\beta_m} = 13$ mV) Abscissae changes of B_{α_m} and B_{β_m} Ordinates (A) rheobases in mA/cm (B) threshold potential (V_t) in mV

Discussion

The problem of accommodation in the myelinated nerve fibre was dealt with in the present investigation The basis for this treatment has been the ionic theory for impulse generation A quantitative form of the theory sufficiently complete for numerical computations is available from the voltage clamp analysis Computations were performed of the response of the model nerve membrane to slowly increasing currents This was done in order to find out how well slow changes in excitability are contained in the quantitative nerve model

The general behaviour of the nerve model in response to stimulating currents of long duration will first be considered in relation to the previous experimental findings then some quantitative details will be dealt with

The nerve model according to the standard data list gave rise to repetitive action potentials of apparently infinite number when step current stimulation of long duration was used A slight modification of the data list in the direction of making the nerve model faster accommodating changed the response to a

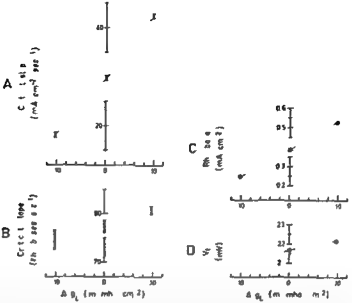

Fig 10 Effect on critical slope rheobase and threshold potential (V_t) of changes in the leak conductance (g_L) Standard data except E_{α_h} = − 22 mV and variation in the leak conductance from its standard value (30 3 m mho/cm) Abscissae changes of g_L. Ordinates (A) critical slope in units of mA/cm /sec, (B) critical slope in units of rheobases/sec (C) rh obase in ~mA/ cm (D) threshold potential (V_t) in mV

The leak conductance (g_L) was varied from 20 0 to 40 0 m mho/cm² in the computations The mean of the leak conductances measured on nerve fibres is 30 3 m mho/cm² (Frankenhaeuser 1960) and 20 9 ± 7 0 m mho/cm² (mean and S D) (Vallbo 1964 b) The changes of g_L (Fig 10) affected the critical slope moderately as measured in units of mA/cm²/sec but the effect was con siderably less on the critical slope as measured in units of rheobases/sec

Effects on rheobase and threshold potential of changes in the constants

The effects on the rheobase and the threshold potential (V_t) of changes in the various constants are shown in most of the above graphs and in Fig 11 These thresholds as measured by a step current of one msec duration were not much affected by most of the changes The exceptions were that a shift of the rate constants α_m and β_m was associated with a marked change of the rheobasic current and the threshold potential The latter changed by about the same amount as the shift of the two constants along the potential axis (Fig 11) A change of g_L had a marked effect on the rheobasic current, about propor tional to the change in g_L but the effect on the threshold potential was negligible (Fig 10)

stimulating current increased slowly This is probably associated with the fact that the membrane potential during the action potential did not reach values close to the sodium equilibrium potential and thus the decrease in the electrochemical driving force for the sodium current was not nearly as marked in this case as when an action potential was elicited by a step current The leak current was by far the largest ionic current before regeneration occurred during stimulation by slowly rising currents (see Table I)

The computations correspond to the experimental situation in which the longitudinal current is zero i e the load seen by the membrane is zero It seems clear that the membrane during activity elicited by slowly rising currents must be more load sensitive than the membrane when stimulated by step currents A much smaller sodium carrying capacity is available for the regenerative response because most of the sodium mechanism is inactivated by the slowly increasing current

One aim of the present investigation was to elucidate the problem concerning which factors account for the normal variation in accommodation among the myelinated nerve fibres from the clawed toad The most attractive and straightforward way to tackle this problem would clearly have been to do a complete voltage clamp and excitability analysis of a number of single nerve fibres The data so obtained could then be treated numerically However this has been too difficult to achieve The standard data list used by Frankenhaeuser and Huxley (1964) was therefore used as the basis for the present computations This data list which is lumped together from various experiments might not describe a fibre with average properties The present computations together with experimental findings (Vallbo 1964 b) showed also that the standard data list describes a fibre with a slower accommodation than is usual for the fibres from *Xenopus laevis* (see Fig 6) The experimentally determined threshold potential (28 5 ± 3 0 mV mean and S D) was somewhat higher than the threshold potential of the standard nerve model (20 4 mV) This finding and further considerations concerning the deficiencies in the voltage clamp technique (summarized by Frankenhaeuser and Huxley 1964) suggest that the known errors in the voltage clamp analysis might well be sufficient to explain the discrepancies between the measured and the computed critical slopes These errors seem to be equivalent to about 3—5 mV in the constants B and are likely to be somewhat larger for the m variable than for h n and p The computations of the critical slopes and the response of the nerve model to stimulation by step currents of long duration indicated in two different ways that the nerve model according to the standard data list needs very little modification to respond with the same slow excitability changes as the real nerve fibre

The computations indicated that almost every constant in the data list to some extent affected the critical slope A change which decreased the outward ionic current or increased the inward ionic current at potentials close to the threshold potential made the accommodation slower and a change which increased

the outward or decreased the inward ionic current, made the accommodation faster However prediction of accommodation from the computed steady state currents and permeabilities was not particularly successful The effect of variation in the leak conductance on the critical slope as measured in units of rheo bases/sec was thus surprisingly small A change of the sodium and potassium permeability constants changed the critical slopes as expected in opposite directions, but by about the same amount in the sense that a change of one by a certain factor had about the same effect as a change of the other by the same factor Over a limited region the effect of changes in the inactivation of the sodium permeability was of the same order of size as the effect of a corresponding change of the turning on of the potassium permeability *i e* equal shifts of the $h_\infty - V$ and $n_\infty - V$ curves had the same effect The inactivation had however a considerably greater effect when the $h_\infty - V$ curve was shifted sufficiently much to the right It seemed that it was possible to make the nerve model quite fast accommodating by changing the potassium permeability in one direction but not always to make it very slowly accommodating only by changing the potassium permeability in the other direction Changes within the experimentally found range of the inactivation were associated with computed accommodations almost covering the range of accommodations found experimentally A very small change of the inactivation by a shift of the $\alpha_h - V$ relation to the right by only a few mV from the standard curve would probably give a nerve model with infinitely low critical slope

The computed rheobase of the standard fibre was 0 36 mA/cm² while the rheobase as measured from the nerve fibres was 0 22 mA/cm² (mean) This difference might largely be attributed to the difference in leak conductance which was 30 3 m mho/cm² in the standard nerve model and 20 9 m mho/cm² in the nerve fibres investigated by Vallbo (1964 b) The computations indicated that the rheobasic current was nearly proportional to the leak conductance The threshold potential in the nerve model according to the 'standard data list was 20 4 mV and that of the nerve fibres was 28 5 mV (mean) This deviation might be caused by the known errors in the voltage clamp method

The agreement between the findings obtained from the experiments on myelinated nerve fibres and those obtained from the computations presented here seems to be good enough to allow a discussion of the specific problem concerning which factors account for the normal variation in accommodation among the myelinated nerve fibres A close relation has been found experimentally (Vallbo 1964 b) between the critical slope and the inactivation of the sodium mechanism (h) The critical slope has not been found to depend closely on the sodium and potassium permeability constants ($\overline{P}_{Na}$ and P_K) nor on the leak conductance (g_L) No experimental information is available concerning the relation between the critical slope and the turning on of the potassium permeability (n) The results of the computations indicated that the inactivation of the sodium mechanism had a marked effect on the critical slope the turning on of the potas

sium permeability and the two permeability constants had some effect while the leak conductance did not much affect the critical slope It seems therefore likely that the permeability constants and the leak conductance are of minor significance for the normal variation in accommodation that the inactivation of the sodium permeability very likely is the most important factor while the effect of the turning on of the potassium permeability cannot be entirely neglected

The present investigation indicates that the voltage clamp analysis accounts reasonably well for the accommodation and even for variations in accommodation among the fibres The agreement between the experimental findings and the results obtained by the computations was by no means perfect However the agreement seems surprisingly good in view of the large difficulties to extract experimentally the rate constants in the potential range ($0 < V < 20$ mV) which is critical for the accommodation It is quite clear that although there is no simple way to account for accommodation the accommodation is satisfactorily included in the equations for the ionic currents The time constant of accommodation (λ) (*e g* Katz 1939) has been used to describe accommodation and concepts of this kind seem useful for studying excitability However it seems rather meaningless to use such a simplification when the physical basis of accommodation is considered because this basis is clearly of an extremely complex nature

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On the Bile Acid and Neutral Fecal Steroid Excretion in Man and Rabbits Following Cholesterol Feeding

Bile Acids and Steroids 150

By

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Abstract

Hellström K. *On the bile acid and neutral fecal steroid excretion in man and rabbits following cholesterol feeding* Acta physiol scand 1965 *63* 21—35 The effect of cholesterol feeding on bile acid turnover and neutral fecal steroid excretion was studied in rabbits and human subjects In rabbits the addition of 1 cholesterol to a low fat pellet diet (on the average 700 mg of cholesterol per day) caused a marked increase of the serum cholesterol level but a reduction of the turnover and the fecal elimination of deoxycholic acid On the subsequent feeding of a semisynthetic cholesterol free diet the serum cholesterol level decreased in spite of a further reduction of the fecal bile acid excretion When human subjects received a diet containing egg yolk lipids which provided 1 4 g cholesterol per day the serum cholesterol level increased moderately over the control values but there were no consistent effects on either the biliary bile acid composition or on the turnover of cholic acid The amount of cholesterol excreted as neutral fecal steroids during the cholesterol feeding period corresponded on the average to 80 and 77 % of the amount of cholesterol given to rabbits and human subjects respectively Cholesterol and the plant sterols β sitosterol and methylcholesterol were metabolized into neutral compounds that were the same in rabbit and in human feces Rabbit feces contained proportionally more 5α sterols than human feces

Different species do not react in the same manner to cholesterol feeding Rabbits and chickens become severely hypercholesterolemic whereas with dogs and rats the influence of ingested cholesterol is much less marked (Katz and Stamler 1953) Human subjects respond to cholesterol (egg yolk) supplemented diets with a moderate increase in the serum cholesterol level (Messinger *et al* 1950 Beveridge *et al* 1960 and Connor *et al* 1961) The mechanism for the regulation of the serum cholesterol level and of the distribution of cholesterol in the body is not well enough understood to permit a complete explanation of these species differences It has been shown that cholesterol synthesis in extra

hepatic tissues is almost uninfluenced of cholesterol feeding (Gould *et al* 1953, Cox *et al* 1954) whereas in man as well as in experimental animals the hepatic cholesterol synthesis is promptly suppressed (Gould 1951, Tomkins *et al* 1953 Frantz *et al* 1954, and Bhattathiry and Siperstein 1963) Differences in the activity of this feedback mechanism may, at least in part be responsible for the variations in response to cholesterol feeding observed among species

Another mechanism which tends to counterbalance changes in the body cholesterol pool has been demonstrated in dogs and rats (Abell *et al* 1956 Wilson 1962) These animals were found to have an increased elimination of cholesterol as fecal bile acids when cholesterol rich diets were fed Since the bile acid excretion represents a considerable part of the cholesterol that is eliminated from the body and since the changes were highly significant (100—200 %) alterations in bile acid excretion seem to be an important compensatory mechanism for the absorption of increased amounts of dietary cholesterol

In view of the differences in susceptibility to hypercholesterolemia induced by cholesterol feeding apparent when dogs and rats are compared to rabbits and probably to man it was of interest to determine whether the last two species when faced with an excessive dietary cholesterol intake could also eliminate increased amounts of cholesterol as bile acids Due to the lack of reliable methods for quantitative analysis of the total fecal bile acids bile acid metabolism was studied by using a tracer technique with C^{14} labeled bile acids characteristic of the different species A standardized diet was fed which was subsequently supplemented with cholesterol As an extension of an earlier study on the effect of saturated and unsaturated fat and type of diets on the bile acids ...bolism in the rabbits (Hellstrom *et al* 1962) some animals were also studied after being transferred from the cholesterol supplemented diet to a semisynthetic diet in which corn oil was one of the main constituents

Experimental

Rabbit experiment Adult male albino rabbits weighing between 2 and 3 kg were used The animals were kept in single hutches from which urine and feces could be collected separately Each rabbit was provided with a stomach fistula through which bile samples were collected by introducing a tube into the duodenum The fistula which consisted of a polyvinyl chloride plastic tube was closed with a stopper and was opened only when duodenal bile samples were collected Details about fistula construction and bile sampling technique have been given in a previous paper (Hellstrom and Sjovall 1962

The rabbits were fed the control diet the control diet supplemented with cholesterol and finally a semisynthetic diet The control diet with a caloric value of 2 5 cal/g and a fat protein and carbohydrate content of 3 5 16 5 and 49 3 % by weight respectively was the conventional low fat commercial pellet ration used in the investigation mentioned above Addition of cholesterol to the control diet was carried out in the following manner Pellets were partly crushed in a mortar and transferred onto a tray Cholesterol dissolved in a volume of ether large enough to cover the crushed pellets was added and the mixture was stirred thoroughly the ether was evaporated at room temperature

and the dry diet was stored at — 20 C until used The semisynthetic diet was prepared from corn oil casein sucrose glucose salts vitamins and cellulose Corn oil constituted 26 % of the calories (For details see Wigand 1959) The consumption of the first diet was adjusted to maintain a constant body weight Subsequent diets were given in isocaloric amounts About 65—80 g per day of the control and cholesterol supplemented diets and 48—60 g of the semisynthetic diets were given to the rabbits

The control diet was given to the rabbits for several weeks before the experiment started Two weeks after the production of the stomach fistula the rabbits were given deoxycholic acid 24 C intraperitoneally Bile samples were taken every 4 to 7 days for a period of one month, and the turnover of deoxycholic acid was determined Cholesterol was then added to the diet and three to four weeks later a new dose of labeled material was administered When a sufficient number of bile samples had been taken to permit the determination of the deoxycholic acid turnover rabbits 27 B and D were transferred to the semisynthetic diet and the collection of bile samples was continued for a further period of about two months

Feces were collected daily during the whole experiment and were stored at — 15 C until analyzed The urine was rinsed off the hutch tray with ethanol each day and pooled in 3—7 days portions Blood samples were taken from the marginal ear vein

Human experiment The subjects examined were 3 female cadets (age 20—22 years body weights 60—66 kg) from the Salvation Army School in Stockholm They were living at the School and were occupied with their ordinary work.

During the whole experiment each subject was served 4 meals a day The breakfast and supper consisted of coffee or tea toast and marmalade For lunch and dinner the subjects consumed half of a formula portion and fruit The daily intake of bread marmalade and fruit was approximately equal for each subject The intake of the formula ration was adjusted so as to keep the body weight constant The subjects were instructed to consume each diet portion completely

The formula diet was prepared from margarine (purchased from AB Sunco Stockholm) skimmed milk powder (kindly supplied by AB Mjolkcentralen Stockholm) and water One day s portion of this diet had the following composition Margarine 80 g skimmed milk powder 200 g and water 300 ml These components were homogenized with an Ultra Turrax homogenizer (Janke Kunkel i Bra West Germany) until an uniform emulsion was obtained The prepared portions were stored at — 20 C until used

During the second period of the experiment part of the margarine was replaced by egg yolk lipids The egg lipids were obtained in the following manner Pooled egg yolks were homogenized with the homogenizer in an excess of ethanol for five minutes whereafter the mixture was filtered The alcoholic solution was poured onto trays and the ethanol was evaporated at 30 C in a drying oven The batches of egg lipid were pooled and stored at — 20 C until used A daily portion of the formula diet in which egg lipids were included had the following composition Egg lipids 25 g margarine 68 g skimmed milk powder 200 g and water 300 ml

The intake of cholesterol with the control diet was about 50 mg/day The margarine was free of cholesterol the skimmed milk powder contained 4 mg cholesterol per 10 g which corresponded to a daily intake of 20 mg Analyses of bread marmalade and fruit revealed that an approximate figure for the cholesterol intake from these constituents should amount to 30 mg/day

The concentration of cholesterol in the egg lipids was determined in the following way An aliquote was saponified in 1N KOH in 50 aqueous ethanol (1 ml per mg dry weight of the extract) at 60 C for one hour The hydrolysate was extracted three times with petroleum ether the organic phases were pooled washed with 50 % aqueous

ethanol in water until neutral and then taken to dryness under a stream of nitrogen. The cholesterol content in this fraction was determined by GLC (see below). The remaining water phases were combined, acidified to pH 3 with 6N HCL and extracted three times with ether. The organic phases were combined, washed with small volumes of water until neutral and then dried over Na_2SO_4. The dry weight of this fraction, which contained the fatty acids from the aliquot analyzed was determined. These analyses revealed that 25 g of the dried egg yolk lipids contained 1.4 g cholesterol and 12 g fatty acids.

After ten days on the control diet the subjects received an oral dose of C^{14}-labeled cholic acid and its turnover was studied during the next two weeks. Egg lipid diet was then commenced, and two weeks later the subjects were given a new dose of C^{14}-labeled cholic acid, and its turnover was studied again.

Feces were collected continuously after the administration of the first dose of cholic acid except for an interruption during the week preceding the start of the second turnover study.

Determination of bile acid turnover. The radioactive bile acids used had been prepared in this laboratory according to Bergström *et al.* (1953). Cholic acid-24-C^{14} 5 μC was given orally to the subjects and deoxycholic acid-24-C^{14} 13.5 μC was administered i.p. to the rabbits. Both acids were given as sodium salts in saline. Bile samples were obtained by duodenal intubation. An i.v. dose of cholecystokinin (kindly supplied by Prof. E. Jorpes and Dr. V. Mutt, Karolinska Institutet) was given to obtain a free flow of bile. About 15 ml of concentrated bile were collected from the subjects and about 0.5 ml from the rabbits.

Analytical procedure

Determination of specific activity of bile acids

Deoxycholic-24-C. The bile samples were subjected to quantitative paper chromatography (Sjövall 1959). For determination of radioactivity the paper zone containing the labeled bile acid was cut out and placed directly in scintillation vials which were counted in a Packard Tri Carb scintillation spectrometer as described previously (Hellström, Sjövall 1962).

Cholic acid-24-C^{14}. The bile samples taken from subjects I. H. and U. K. were analyzed as described by Lindstedt (1957). The bile was hydrolyzed and the cholic acid isolated by reversed phase partition chromatography using phase system C (Norman 1953). It was crystallized from ethyl acetate and the isotope content was determined according to Glascock (1954). These analyses were kindly performed by Dr. S. Lindstedt.

Cholic acid in the bile samples from subject M. R. was isolated as described above and analyzed quantitatively with GLC (see below). The radioactivity was determined in a Packard Tri Carb scintillation spectrometer.

Analysis of bile acids in human bile

The bile was diluted with ethanol and the precipitate filtered off. The solution was evaporated to dryness under reduced pressure and saponified with 2 N sodium hydroxide for 6 hours at 120° C. The free bile acids were extracted with ether; the ether extracts were washed with small volumes of water until neutral and then evaporated to dryness. An aliquot was dissolved in ether-methanol 9 : 1 and methylated with freshly prepared diazomethane. Quantitative analysis of this fraction was performed by GLC according to the method developed in this laboratory (Eneroth *et al.* 1964 a). Equipment and standard conditions were essentially as described by Sjövall (1962). Biological samples were dissolved in acetone prior to gas chromatography and 4 μl portions, approximately corresponding to 30 μg of the extract, were injected with a 10 μl Hamilton syringe. Peak areas were measured by planimetry and compared with those obtained for known

amounts of appropriate reference standards A linear response was found for all bile acids within the limits used for quantitative analyses of bile acids (0 5—5 μg) in biological extracts

Analyses of feces

Feces collected over 3—7 days were pooled homogenized for five minutes in chloroform methanol 1 1 (v/v) with an Ultra Turrax homogenizer and then extracted continuously for 48 hours with the same solvent mixture in a Soxhlet extractor of siphon type The extracts were stored at room temperature in dark tightly stoppered bottles until analyzed (within 1—30 days)

Determination of bile acids in rabbit urine and feces The radioactivity in extracts of feces and urine were determined by wet combustion according to the method of Jeffay and Alvarez (1961) as described earlier (Hellstrom and Sjovall 1962)

The mean daily excretion of bile acids was calculated by dividing the radioactivity excreted with the mean specific activity of the biliary glycodeoxycholic acid during the corresponding period

Determination of neutral fecal steroids An aliquot of the chloroform methanol extract was refluxed for one hour in 1N NaOH in 50 % aqueous ethanol (0 1 ml per mg dry weight of the extract) The steroids were then extracted with petroleum ether and an aliquot of the extract was analyzed by GLC as described recently (ENEROTH *et al* 1964 b)

Bile acids in rabbit blood

Heparinized blood (3—5 ml) was added dropwise to 100 ml of ethanol After heating for one hour on a water bath the extract was filtered and evaporated in the flasks in which the wet combustion was carried out

Serum cholesterol

Blood samples were taken every week from the human subjects and every second week from the rabbits The serum cholesterol was determined with the Tschugaeff colour reaction The extraction procedure of FOLCH *et al* (1957) was used These analyses were kindly performed by Dr S Lindstedt

Results

Differences were observed in the metabolism of deoxycholic acid by the rabbits on the various diets During the first 14 days after the shift from the control to the cholesterol supplemented diet no changes could be detected either in the fecal elimination of deoxycholic acid and its metabolites or in the fall of the specific activity of the biliary glycodeoxycholic acid However when the turnover study was repeated after 3—4 weeks of cholesterol feeding the half life of deoxycholic acid had increased and the turnover decreased in all rabbits whereas less marked changes had occurred in the pool size (Table I) The mean values for the half lives on the control and the cholesterol supplemented diet were 5 6 and 7 5 days respectively and for the turnover 85 4 and 58 4 mg/day respectively

The mean value for the urinary elimination of deoxycholic acid was unchanged after the addition of cholesterol to the diet but the mean fecal elimination decreased from 68 to 38 5 mg/day in the three rabbits studied (Table II)

Table I Turnover of deoxycholic acid in rabbits on different diets

Rabbit	Diet	No of days on diet[1]	Half life (days)	Pool size (mg)	Production (mg/day)	Serum cholesterol (mg/100 ml)[2]
L	Control diet	10	6.5	510	55	58
	Control diet + 1 % cholesterol	27	9.2	491	37	1 8[illegible]9
G	Control diet	25	4.0	508	88	34
	Control diet + 1 % cholesterol	29	4.5	412	63	1 153
27	Control diet	17	9.8	83[illegible]	59	33
	Control diet + 1 % cholesterol	37	13.0	793	4[illegible]	1 383
	Corn oil	—	17.0	—	—	678
						[4](1 159–200)
B	Control diet	21	4.0	598	104	[illegible]
	Control diet + 1 % cholesterol	[illegible]8	4.5	588	91	1 040
	Corn oil	—	[3]19.0	—	—	590
						[4](720–400)
D	Control diet	[illegible]1	3.6	628	121	69
	Control diet + 1 % cholesterol	25	6.4	5[illegible]	59	812
	Corn oil	—	24.0	—	—	45[illegible]
						[4](600–370)

[1] At the beginning of the turnover study

[2] Mean during each dietary period

[3] Half life of the specific activity of biliary glycodeoxycholic acid when the cholesterol diet had been replaced by the corn oil diet

[4] The values within paranthesis represent the first and the last analyses performed during the corn oil diet period

Table II Comparison between the daily production of deoxycholic acid calculated from half life and pool size and the mean daily excretion of labeled bile acids in feces and urine (mg/day)

Rabbit	Control diet			Control diet + 1 % cholesterol			Corn oil diet	
	Calculated production[1]	Calculated fecal excretion[2]	Calculated urinary excretion[2]	Calculated production[1]	Calculated fecal excretion[2]	Calculated urinary excretion[2]	Calculated fecal excretion[2]	Calculated urinary excretion[2]
27	59.0	41.[illegible]	[illegible]8	4[illegible].0	28.5	5.7	19.1	6.[illegible]
B	104.0	[illegible]5.0	7.0	91.0	[illegible]0	[illegible]	11.3	8.1
D	121.0	88.0	[illegible]2	[illegible]9.0	3[illegible].0	9.[illegible]	[illegible].9	[illegible]

[1] Based on measurements of pool size and half life

[2] Total daily radioactivity in fecal or urinary bile acid fraction divided by the specific radioactivity of biliary deoxycholic acid

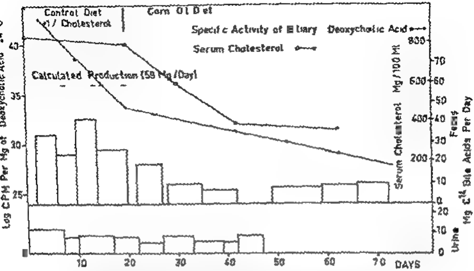

Fig 1 Semilogarithmic plot of the specific activity of biliary glycodeoxycholic acid fecal and urinary excretion of isotope and changes in serum cholesterol level during the period when the cholesterol supplemented diet was replaced by the semisynthetic diet (rabbit D)

When the cholesterol supplemented diet was replaced by the semisynthetic diet there appeared to be an immediate effect on the fall of the specific activity of the biliary glycodeoxycholic acid (Fig 1) The slope of the line obtained by plotting the logarithm of the specific activity versus time became less pronounced The urinary elimination of deoxycholic acid remained unchanged whereas the fecal excretion was further reduced The ratio between the mean fecal elimination of deoxycholic acid on the control diet the cholesterol diet and the semisynthetic diet was approximately 5 3 1

The excretion of radioactive compounds in feces and urine did not account for all of the daily elimination of isotope as calculated from the turnover of deoxycholic acid The mean recovery in rabbits 27 B and D on the control diet was 79 % and on the cholesterol diet 76 %

The concentration of bile acids in the blood of different rabbits ranged between 0 42—9 30 mg per 100 ml (Table III) but large variations were observed even in the same rabbit when examined at different times No consistent differences could be related to changes of diet

The neutral fecal steroids found in rabbit feces were cholesterol and its metabolites (coprostanol cholestanol and coprostanone) the two plant sterols β sitosterol and metylcholesterol and their corresponding metabolites Coprostanone 24β ethyl and methylcoprostanone were only present in trace amounts The excretion of cholesterol and cholestanol was almost similar on the control and the corn oil diets whereas on the latter as well as on the cholesterol supplemented diet proportionally smaller amount of cholesterol had been transformed

Table III Concentration of bile acids in rabbit blood expressed as mg of deoxycholic acid per 100 ml of whole blood

	Control diet		Control diet + 1 % cholesterol	
Rabbit	No of samples	Bile acids (mg/100 ml)	No of samples	Bile acids (mg/100 ml)
B	1	0.69	2	0.77 (0.72–0.82)
D	1	0.42	3	3.00 (1.30–4.90)
27	1	1.99 (0.87–3.10)	2	0.92 (0.9[illegible]–0.91)
L	2	3.60 (2.50–4.80)	4	4.70 (2.20–9.30)
G	1	0.44	—	—

Table IV Mean excretion of neutral fecal steroids in rabbits on different diets (mg/day)

Rabbit	Diet	Coprostanol	Cholesterol	Cholestanol	Cholesterol eliminated as neutral steroids
I	Control diet	2.3	13.6	5.6	21.5
	Control diet + 1 % cholesterol	12.9	443.0	[3]8	463.9
	Corn oil	1.4	11.[illegible]	6.3	19.3
B	Control diet	10.9	5.1	3.9	19.9
	Control diet + 1 % cholesterol	125.0	423.0	4	552.0
	Corn oil	1.3	7.3	2.8	11.4
[illegible]	Control diet	9.2	9.2	2.9	21.3
	Control diet + 1 % cholesterol	209.0	328.0	6	543.0
	Corn oil	0.5	5.2	3.8	9.5
Mean	Control diet	7.5	9.3	4.1	20.9
	Control diet + 1 % cholesterol	115.6	398.0	6	519.6
	Corn oil	1.1	8.0	4.3	13.4

[1] Feces collected over 2–6 weeks during each dietary period was analyzed
[2] Calculated as the sum of cholesterol coprostanol and coprostanone
[3] Approximative figure (incomplete separation between cholesterol and cholestanol)

into coprostanol The mean ratios between cholesterol and coprostanol on the control diet the cholesterol supplemented diet and the corn oil diet were 1.2 3.4 and 7.3 respectively

Table 1 Excretion of neutral fecal steroids in the human subjects (mg day)

Subject	Diet	No. of samples analyzed	Days[1]	Coprostanol	Cholesterol	Coprostanone	Cholesterol eliminated as neutral steroids[2]	Cholesterol intake minus output[3]	β ethyl coprostanol	β sitosterol	Methyl coprostanol
UK	Control diet	3	8	377 (504–68)	112 (230–68)	48 (117–4	537	–487	28 (65–11)	25 (30–15)	III (3–16)
	Egg lipid diet	3	15	869 (1,309–488)	289 (314–258)	37 (48–16)	1 190	260	43 (53–37)	14 (16–12)	29 (35–16)
MR	Control diet	3	11	305 (332–185)	41 (59–24)	14 (70–8)	360	–310	54 (68–38)	4 (7–4)	23 (31–15)
	Egg lipid diet	3	12	693 (730–460)	91 (111–27)	34 (44–15)	818	632	35 (39–31)	7 (8–6)	14 (19–4)
IH	Control diet	3	12	323 (567–156)	33 (150–18)	27 (88–11)	3 8	–328	63 (75–33)	11 (21–8)	30 (43–17)
	Egg lipid diet	3	13	1 056 (1 144–902)	92 (98–83)	193 (246–158)	1,341	109	102 (124–82)	15 (17–12)	81 (93–62)
Mean on the control diet				335	62	28	425	–375	48	13	24
Mean on the egg lipid diet				873	157	86	1 116	333	60	12	41

[1] Duration of feces collection
[2] Calculated as the average of sums of cholesterol coprostanol and coprostanone
[3] Daily cholesterol intake minus cholesterol eliminated as neutral fecal steroids

As was the case with the bile acids the excretion of neutral fecal steroids of endogenous origin was smaller on the corn oil than on the control diet The mean excretion of these two diets was 20 9 and 13 4 mg per day respectively The amount of cholesterol excreted as neutral fecal steroids on the cholesterol supplemented diet amounted approximately to 67 85 and 84 % of the cholesterol added to the diet given to rabbits 27 B and D respectively

Human subjects The effect of the egg lipid diet on the serum cholesterol level in the human subjects was not as marked as the effect brought about by the

Table VI Turnover of cholic acid in the human subjects

Subject	Diet	Half life (days)	Pool size (mg)	Daily production (mg/day)	C CD D[1]	Serum cholesterol (mg/100 ml)[2]
UK	Control diet	2.8	1 650	410	1 58 1 0.27	245
	Control diet + egg lipids	1 9	1 090	394	1 20 1 0 16	281
MR	Control diet	2 2	1,240	390	2 60 1 0 40	133
	Control diet + egg lipids	4 5	1,582	255	5 60 1 0 45	203
IH	Control diet	3 0	1,900	265	1 46 1 0.27	218
	Control diet + egg lipids	4.5	2 105	375	1 43 1 0 47	270

[1] Mean ratio between cholic chenodeoxycholic and deoxycholic acid in duodenal bile samples
[2] Mean during the period.

cholesterol supplemented diet in the rabbits (Table VI and I) However the serum cholesterol level increased in all subjects during the second dietary period (mean increase 30 4 %) The values given in Table V for the excretion of neutral fecal steroids represent the mean excretion during the time when the turnover of cholic acid was studied In addition to cholesterol and its metabolites coprostanol and coprostanone small amounts of plant steroids (β sitosterol 4β-ethylcoprostanol and methylcoprostanol) were found After addition of egg lipids to the diet the excretion of cholesterol and its neutral metabolites increased in all subjects on an average by 168 % but there was very little change in the proportional contribution of the different cholesterol metabolites When only the elimination of cholesterol as neutral steroids was taken into account the subjects were in a negative cholesterol balance during the control period and in a positive balance during the second dietary period The mean difference between intake of cholesterol and output as neutral fecal steroids on the two diets were — 375 and + 333 mg per day respectively

The main bile acids found in the human bile were cholic acid chenodeoxycholic acid and deoxycholic acid The ratios between these acids were similar in subjects U K and I H on both diets whereas in subject M R the cholic acid increased proportionally on the egg lipid diet (Table VI) In addition to above mentioned acids trace amounts of lithocholic acid and smaller amounts of ursodeoxycholic acid were found in most bile samples

When the control diet was replaced by the egg lipid diet the turnover of cholic acid increased in subject I H decreased in subject M R and remained almost unchanged in subject U K The half life and pool sizes of cholic acid were not the same during the two dietary periods in any subject There were no consistent changes that could be related to the changes of diet (Table VI)

Discussion

The turnover of deoxycholic acid in rabbits fed different diets has been studied earlier in this laboratory (Hellstrom *et al* 1962) It was then found that semisynthetic diets when compared to the control diet caused a longer half-life a smaller turnover and a reduced fecal elimination of deoxycholic acid whereas the urinary excretion of bile acids was unchanged The changes observed were of the same order of magnitude whether the dietary fat was hydrogenated coconut oil or corn oil Since a reduced fecal excretion of bile acids was apparent immediately on transferring rabbits to the semisynthetic diet it was suggested that the primary action of this diet might be on the intestinal absorption of bile acids and that the synthesis of these compounds was depressed secondarily

The changes in the deoxycholic acid turnover observed in the present study when cholesterol was added to the control diet were similar in some respects to those found earlier on replacing the control diet with semisynthetic diets In both cases the deoxycholic acid turnover and the fecal bile acid excretion were reduced however the cholesterol diet had only a moderate effect on the half life of deoxycholic acid Contrary to the rapid initiations of changes in the bile acid metabolism effected by the semisynthetic diets the effects of the cholesterol diet were not apparent during the first 14 days after the start of the diet This could indicate that the cholesterol supplemented diet did not influence primarily the bile acid absorption but that the changes in the deoxycholic acid metabolism were due to an effect of the cholesterol diet on the liver These changes might be correlated with the development of a fatty liver which has been encountered as a frequent complication of cholesterol feeding in rabbits (Chalatow 1914)

When the cholesterol supplemented diet was replaced by the semisynthetic diet the fecal elimination of deoxycholic acid as well as the fall of the specific activity of glycodeoxycholic acid in the bile was further reduced (Fig 1) These changes were quantitatively similar to those observed in the previous investigation when rabbits were transferred directly from the control to the semisynthetic diets The mean fecal elimination of deoxycholic acid (for 5 rabbits) was then calculated to be 19 3 mg per day In the present study the corresponding value on the same semisynthetic diet was 13 1 mg per day

It has been repeatedly reported that the decrease of the serum cholesterol usually found when saturated fat is replaced by unsaturated fat in the diet is accompanied by an increased excretion of fecal bile acids in man (Gordon *et al* 1957 Haust *et al* 1958 and Goldsmidt *et al* 1960) and in rats (Merrill 1959 Nath and Brahmankar 1961) In the previous investigation in rabbits we found no correlation between the changes in the serum cholesterol level induced by different dietary fats (corn oil and hydrogenated coconut fat) and the turnover of deoxycholic acid In the current study the serum cholesterol fell markedly in the rabbits during the corn oil period and the fecal bile acid excretion decreased simultaneously

From work outlined in a previous report (Hellström *et al* 1962) it was concluded that the deoxycholic acid production represents the main part of the cholic acid synthesized in the rabbit and that variation in the deoxycholic acid production seemed to be representative of the total bile acid production in the liver The composition of the biliary bile acids found in this study (glycodeoxycholic and trace amounts of free deoxycholic acid) did not indicate that the reduced synthesis of deoxycholic acid during the cholesterol and corn oil diet period was compensated for by an increased synthesis of other bile acids

The human subjects studied in the present work had values for half lives pool sizes and turnover of cholic acid which were in good agreement with those earlier reported by Lindstedt (1957) for normal students on a regular diet In the 3 subjects no consistent changes in the cholic acid turnover or in the composition of the biliary bile acids could be related to the change of diet Thus there are no indications from the results obtained in the present study in rabbits and human subjects that cholesterol feeding, under the conditions used resulted in an increased bile acid excretion

In contrast to these results earlier studies in rats by Wilson (1962) and in dogs by Abell *et al* (1957) have shown a marked increase in the excretion of fecal bile acids after supplementation of 1 % cholesterol to the diet However due to differences in the experimental techniques used in the abovementioned and in the present studies the result cannot be used directly for comparisons of the abilities of the different species to handle excessive loads of dietary cholesterol For instance the human subjects in this study were fed much smaller amounts of cholesterol per kilogram body weight than the different animals studied Furthermore the rats studied by Wilson were only fed cholesterol for 5 days prior to the measurements of the bile acid excretion and the bile acid analyses made by Abell *et al* were based on non isotopic methods Despite these experimental differences it seems probable that rats and dogs in contrast to rabbits and possibly to humans are able to counteract the hypercholesterolemic effect of cholesterol feeding by accelerating their bile acid formation and excretion

In a recent report from this laboratory (Eneroth *et al* 1964 b) it has been demonstrated that the neutral metabolites of the plant sterols β sitosterol and methylcholesterol found in human feces are analogous to the metabolites of cholesterol i e to coprostanol, coprostanone and cholestanol

Gas chromatographic analyses revealed that the abovementioned neutral steroids were all present in rabbit feces which, when compared with human feces contained proportionally larger amounts of cholestanol and its analogues of plant sterol origin whereas coprostanone was of minor importance quantitatively

The mean excretion of cholesterol coprostanol and cholestanol in the rabbits during the control period was 21 1 mg/day which is less than the excretion of digitonin precipitable sterols (50 mg per day) earlier reported for rabbits on a sterol free diet (Mosbach *et al* 1956) It is interesting to note that the excreted amount of neutral steroids originating from cholesterol was approximately

similar in the rabbits and the human subjects during the control period when calculated as mg steroids per kg b w

The daily intake of cholesterol in the human subjects during the cholesterol feeding period was 1450 mg (22 mg per kg b w) The excretion of neutral fecal steroids derived from cholesterol during this period on the average corresponded to 77 3 % of the amount of cholesterol ingested In contrast to the findings in rabbits the ratios between cholesterol and its metabolites (coprostanol and coprostanone) in feces did not increase after the addition of cholesterol to the diet Thus the intestinal microorganisms in man are able to metabolize large amounts of cholesterol This is of interest since the addition of corn oil to a low sterol diet has been found to have an inhibiting effect on the transformation of cholesterol into coprostanol and coprostanone (Eneroth *et al* 1964 b) In that experiment the daily sterol intake (predominantly β sitosterol and methylcholesterol) amounted to 400—500 mg per day It is thus evident that the ingested amount of sterol per se is not responsible for the inhibited cholesterol transformation brought about by the corn oil diet

Cholesterol in the serum originates from dietary and endogenous sources Studies in experimental animals such as rats (Tomkins *et al* 1953 Frantz *et al* 1954) dogs and rabbits (Gould 1951) and chickens (Sakakida *et al* 1963) have demonstrated that cholesterol feeding promptly suppresses the hepatic synthesis of cholesterol whereas the synthesis in the extrahepatic tissues shows little change in response to dietary cholesterol ingested (Gould *et al* 1953 Cox *et al* 1954) The extent of the contribution of hepatic and extrahepatic synthesis to the serum cholesterol varies among species In the rat and in the dog the main part of the nondietary cholesterol of endogenous origin is synthesized in the liver (Eckles *et al* 1955 Hotta and Chaikoff 1955 Taylor *et al* 1960) but in man the cholesterol synthesized in extrahepatic tissues appears to be of considerable importance (Taylor *et al* 1960) It seems conceivable that a proportionally large capacity for cholesterol synthesis in the liver could be correlated with a large capacity to compensate for cholesterol loading It has been suggested that 1 5—2 g is a good approximation of the maximum rate of cholesterol synthesis in intact healthy man and that most of this synthesis occurs in the liver (Gould 1951 Ivy *et al* 1957) In a recent study of Bhattathiry and Siperstein (1963) it was clearly demonstrated that the hepatic synthesis in man was almost completely inhibited when 4 g of cholesterol had been included in the diet for three days It appears that the human liver is capable of compensating for alterations in cholesterol intake by reciprocal changes in the synthesis of cholesterol The maximum capacity for this mechanism can only be as large as the liver s total capacity for cholesterol synthesis

In the present study a completely effective compensation does not appear to have been achieved since the serum cholesterol level increased in all subjects This increase most probably reflected changes in the body cholesterol pool possibly with an accumulation of cholesterol in the liver as has been encountered

in experimental animals after cholesterol feeding (Gould 1951 Katz and Stamler 1953) On the control diet the mean elimination of cholesterol as neutral fecal steroids from the body pool was 375 (range 487—310) mg/day whereas on the egg lipid diet the amount of the cholesterol consumed exceeded the mean elimination as neutral steroids in feces by 333 (range 632—109) mg per day To prevent a change of the body cholesterol pool during the second dietary period the liver had to compensate for 708 (range 942—437) mg per day either by reducing cholesterol synthesis and/or by increasing the elimination of cholesterol as bile acids The result from the present investigation does not indicate any consistent changes in the bile acid formation after addition of the egg lipids to the diet The egg lipid preparation replaced an isocaloric amount of margarine in the diet and providing that the minor differences in the fat composition of the two diets (apart from the cholesterol content) did not markedly influence the cholesterol synthesis the increased cholesterol pool after cholesterol feeding might have either of the following explanations 1 The *feedback mechanism in the liver has its maximal efficiency only when larger* amounts of cholesterol are absorbed and the amount of cholesterol absorbed in this instance was too small to depress the hepatic synthesis completely 2 The liver synthesis was completely depressed but the amount of cholesterol absorbed exceeded the amount synthesized during the control period Under such circumstances this synthesis on the average should be less than 700 mg per day This figure for the hepatic synthesis is lower than the values other investigators arrived at by different types of calculations However the findings by or *et al* (1960) that only a relatively small fraction of the serum cho sterol is of hepatic origin may further indicate that the capacity of the liver to ontrol serum cholesterol level by reducing its synthesis is lower than has ommonly been assumed

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From the University Institute for experimental medicine Copenhagen Denmark

Some Characteristics of the Proximal Tubular Wall Related to Reabsorption During Luminal Occlusion Following Interruption of Glomerular Filtration

By

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Abstract

Leyssac P P *Some characteristics of the proximal tubular wall related to reabsorption during luminal occlusion following interruption of glomerular filtration* Acta physiol scand 1965 *63* 36—45 — The purpose of this investigation was to study the hydrostatic pressure changes within and outside the proximal tubules and to study possible changes in proximal cellular volume during the occlusion period following abruption of the renal blood flow and filtration The pressures were measured directly using the micropuncture technique The results demonstrate that the proximal tubular wall can resist a modest positive hydrostatic pressure from either *the inside or the outside of the cellular wall for a short time interval* During reabsorption in the occlusion period a positive pressure seems to be created in the lumen of the proximal tubules probably due to interfacial tension and a positive luminal pressure is maintained until all the fluid has disappeared

The proximal cellular volume was estimated from the cellular area of cross sectioned proximal tubules of snap-frozen kidneys The area was measured by planimetry It was demonstrated that the cellular area is the same (within about [illegible]) at the various rates of transcellular transport of sodium associated with spontaneous variations of the rate of glomerular filtration and *that it remains unchanged* during the whole occlusion period An increase of about 50 % in the cellular volume occurred as soon as the luminal fluid was reabsorbed i e about 20 sec after interruption of the renal circulation The cellular volume then remained unchanged for several minutes

In a previous study evidence was presented that the proximal tubular reabsorption of filtrate in mammalian kidneys has the character of a T max process and it was concluded that changes in the rate of glomerular filtration were brought about by primary changes in the proximal T max of salt (Leyssac 1963) This concept implies that even such moderate changes in the proximal intratubular pressure which may be caused by primary changes in the rate of reabsorption will significantly influence the rate of filtration as predicted already by Brandt Rehberg in 1929 and further

emphazised by Bojesen (1955 1957) Furthermore it would imply some rigidity of the proximal tubular wall to resist such moderate changes in the luminal pressure induced by variations in the rate of reabsorption However micropuncture studies by Wirz (1955) and by Gottschalk and Mylle (1956) have shown that in the steady states mean pressures in the proximal tubules are identical with the mean peritubular capillary pressures under different conditions and pressures In the present investigation intratubular and peritubular capillary pressures were measured during the short term pressure changes following interruption of the renal blood flow and filtration and it is demonstrated that the tubular wall of the proximal convolutions may resist temporary pressure differences

It was previously concluded that during the luminal occlusion of proximal tubules following abruption of glomerular filtration the reabsorption continued at an unaltered rate until no more fluid was left in the lumen (Leyssac 1963) However an increase in the hight of the cells of proximal tubules with partially occluded lumina (decreased diameters) was observed An increase in the volume of the cells reflecting changes in the intracellular ion composition and water content is a likely consequence of gross interference with the active pump mechanism unless permeability changes occur simultaneously as emphazised by MacRobbie and Ussing (1961) Since changes in the external diameter of the proximal tubules also occurred during the occlusion period changes in the cellular volume could not be evaluated by measuring the cellular thickness Therefore in the present investigation the proximal cell volume was estimated by measuring the cellular area of cross sectioned proximal tubules at different rates of transcellular transport and during the occlusion period The results indicate that the cellular volume is uninfluenced within the error of the method at spontaneously occurring rates of transcellular transport and during the whole occlusion period However as soon as the lumen is emptied of tubular fluid the cellular volume increases

Methods

Male or female albino rats about 250 g body weight were used exclusively The animals were anesthetized with sodium amytal and the left kidney exposed The general set up and technique for micropunctures and measurement of pressures in the tubules and peritubular capillaires of rat kidneys is described in the preceding paper (Leyssac 1964 b) In one group of animals an open clamp was fixed round aorta proximal to the renal arteries in such a way as to permit a total interruption and reestablishment of the aortic circulation with a minimum of movement of the observed kidney By this procedure the micropipette fairly often remained within the lumen of a punctured tubule even during the occlusion of the lumen following interruption of the renal circulation in spite of changes occurring in the volume of the kidney It was thus possible to follow pressure changes within the lumen of proximal tubules after interruption of the renal circulation and filtration This measurement, however turned out to be extremely difficult in cases of peritubular capillaries partly because the micropipette most often was displaced from the small lumen by volume changes of the kidney and partly it seemed because the pressure drop was very rapid and could not easily be followed Successful measurements were obtained from only two capillaries following aortic clamping In another group of animals pressure changes were monitored within the lumen of proximal tubules and peritubular capillaries after intravenous injections of 25 ng of synthetic angiotensin (Val 5-angiotensin II amid CIBA) When more than one dose of angiotensin was administered to the same animal a time interval of at least 10—15 min elapsed between the injections Two or more control pressures were recorded from each lumen before either clamping the aorta or injecting the angiotensin

In one group of animals from another series of experiments clearances of inulin were determined from ureteral urine collections and plasma samples as described previously (Leys

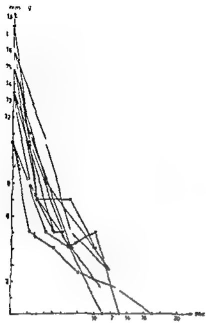

Fig 1 The hydrostatic pressure declines following abrupt clamping of the aorta at zero time Broken lines p ritubular capillaries Full lines proximal tubules

sac 1963) Immediately after the last collecting period the kidney was snap-frozen in a freezing mixture of solid carbon dioxyde and acetone at — 65 C or in isopentane cooled to about — 170 C with liquid nitrogen In another group of animals a ligature was placed loosely round the renal hilar vessels When urine flows were stable above 1 µl/min the ligature was qu ckly tightened and the kidneys frozen at different time intervals from ligation After snap-freezing the tissues were freeze substituted in absolute ethanol embedded in paraffin sectioned and stained as described in a previous paper (Leyssac 1963)

The histological sections of snap frozen cortical tissue were examined in a Reich rt micro scope supplied with a micro projection device the pictures were reflected by means of a pla n mirror m a smooth white paper surface on the table A constant magnification in two per pendicular dimens ons was assertained from day to day by means of a micrometer object The total cellular area of cross sectioned circular proximal tubules were measured by pla nimetry Each tubule was measured twice and 50 superficial proximal tubules were measured in each kidney An encircled standard tubule was measured twice before each new section to assertain a constant magnification from one examined kidney to the next Ten subse quent measurements of the standard tubule gave a mean area of 770 ± 26 µ (stan dard error of the mean) Since changes in the cellular volume are more l kely to manifest themselves in the height of the cells (from peritubular surface to lumen) because of less re sistance m this direction and are less likely to be manifest in a long tud nal direction any change in the cellular volume will be proportional to changes in the cross sectioned area

Results

Fig 1 demonstrates that the pressure in the peritubular capillaries drops to zero immediately following abrupt clamping of the aorta proximal to the renal arteries (broken lines) The fact that zero value is not measured until about 4 sec after clamping is probably due mostly to the technical difficulty of following such a rapid pressure change In the proximal tubules the initial pressure fall is almost parallel to that in

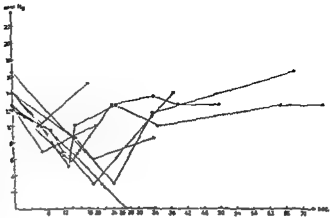

Fig 2 Changes in the proximal intratubular hydrostatic pressure following i v injections of 25 ng syntetic angiotensin

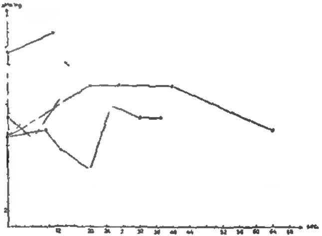

Fig 3 Changes in the peritubular capillary hydrostatic pressure following i v injections of 25 ng syntetic angiotensin

the capillaries However it is apparent that as the capillary pressure reaches zero the proximal cellular wall resists a modest intraluminal positive pressure for a short time interval since the luminal pressure is still 5—7 mm Hg It then gradually declines to zero in a total time of 12—20 sec This change in the slope of the pressure-decline curve would indicate that a positive pressure is built up in the lumen during the occlusion period in spite of the fact that the filtration process has been interrupted and fluid is continuously reabsorbed from the lumen Since 12 –20 sec was exactly the time required to reabsorb the luminal fluid in normal kidneys —the occlusion time (Leyssac 1963) — it may be concluded that a positive intraluminal pressure is maintained until no more fluid is left for reabsorption

This conclusion is further established from the changes observed in the proximal intratubular pressure following an i v injection of a large dose (25 ng) of angiotensin (fig 2) Angiotensin injected i v in such a large dose will constrict the afferent arte

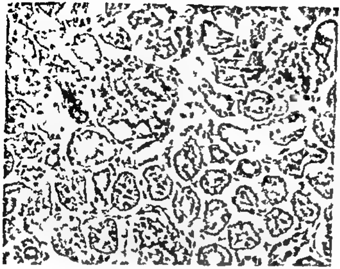

Fig 4 Section (3 μ thick) from the outer cortex of a kidney snap-frozen 18 sec after ligation of the renal artery Most of the proximal tubules are seen occluded Note the displacement of several nuclei to the tubular lumen Magnific × 180

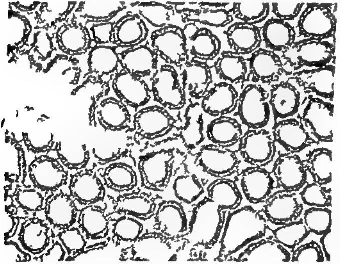

Fig 3 Section (3 μ thick) from the outer cortex of a kidney snap-frozen at the instant of ligation of the renal artery Magnification 180

riole violently as indicated by the paling of the kidney and by the sluggishness of the red cell circulation in the peritubular capillaries even a total disappearance of red cells is often observed here As a consequence the filtration pressure is depressed to such a degree that the process of filtration is impaired However the reabsorption of fluid continues and as is seen from the figure the pressure decline approaches zero in 20—30 sec which was the occlusion time observed after injections of equal amounts of angiotensin (Leyssac 1964 a) The pressure only occasionally actually reaches a zero value because the vasoconstrictive effect is so short lasting that the filtration process is reestablished before the lumen is emptied

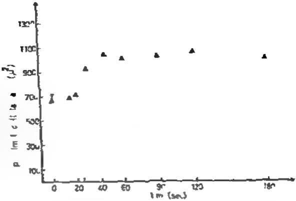

Fig. 6 Changes in the mean cellular area of 50 cross-sectioned proximal tubules of snap-frozen kidneys at different time intervals from ligation of the renal artery (zero time). The vertical broken line represents the end of the occlusion period

In contrast to the inevitable intratubular pressure decline the pressure changes in the peritubular capillaries following similar injections of angiotensin is sometimes seen to increase to a peak, but on other occasions it decreases initially and then increases often to a value above that seen prior to the administration of angiotensin (Fig. 3). Such an increase was never observed in the proximal luminal pressure after angiotensin which would indicate that the rise in the capillary pressure is not transferred to the lumen. These observations demonstrate therefore that the tubular wall (or less likely the thin film of the capillary wall) does resist a modest positive pressure from the outside at least during this short interval of pressure change.

The histological section from a kidney snap-frozen 18 sec after ligation of the renal artery (Fig. 4) shows the morphological appearance of cross-sectioned proximal tubules in a state of function at the very end of the occlusion period immediately before the lumen is emptied. The morphological appearance of cross-sectioned tubules from a kidney snap-frozen at the instant of ligation is shown in Fig. 5. As is apparent from the figures the proximal tubular wall appears "contracted" at the end of the occlusion period, the outer circumference being irregular with several concavities in contrast to the regular circular circumference of the tubules at the initiation of the occlusion period. Furthermore it was regularly observed when the lumen was nearly emptied that nuclei were displaced into the lumen as if they were "squeezed" out of the cells (Fig. 4), which observation is in agreement with the findings of Maunsbach, Madden and Latta (1962) and of Longley and Burstone (1963) in cortical tissue the fixation of which was delayed for a short time. This phenomenon was never seen in sections from kidneys snap-frozen at an earlier stage in the occlusion period.

The cellular area of cross-sectioned proximal tubules is found to be unchanged during the process of transcellular transport in the occlusion period, i.e. within the first 20–25 sec (Fig. 6). Using the cross-sectioned area as a measure of the cellular volume, the observations indicate that the volume of the proximal cells is unchanged during the occlusion period. However, as soon as the lumen is emptied by reabsorption the volume increases considerably (about 50%) and then remains unchanged for several minutes. These observations indicate that during the period of reabsorption of luminal fluid after cessation of filtration the intracellular water and electrolyte content is maintained. However, as soon as there is no further supply of electrolytes and water

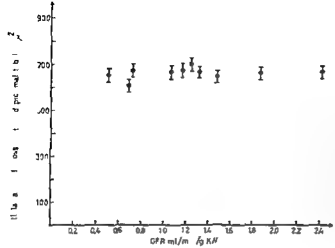

Fig 7 Each point represents the mean cellular area (μ) $\pm$ 2 s e of 50 cross-sectioned proximal tubules from a snap-frozen kidney Glomerular filtration rate (GFR) was determined as clearances of inulin immediately prior to snap-freezing

from the lumen the active pump mechanism is insufficient to maintain a normal intracellular ion composition and the cells consequently swell

Spontaneous variations in the rate of filtration is shown to be without any influence on the cellular area of cross sectioned proximal tubules (Fig 7) These observations demonstrate that in the overall range of filtration rates — corresponding to the rates *of net transcellular sodium transport (STR) (Leyssac 1963) — the cellular volume is* maintained unchanged which indicates that the intracellular water and electrolyte content is not significantly changed

Discussion

It has been established from micropuncture measurements that the mean hydrostatic pressure in the proximal tubular lumina is identical with the mean pressure in the surrounding peritubular capillaries or interstitial space and this identity in pressures is maintained when the pressures are raised considerably by increasing renal venous pressure or ureteral pressure suggesting that an increase in the pressure from outside the tubules would increase the luminal pressure and that an increase in the luminal pressure would be transferred to the interstitial space (Gottschalk and Mylle 1956) The present measurements demonstrate that the proximal tubular wall at least during short term changes in hydrostatic pressures can resist or bear a modest positive pressure from both the outside and the inside of the tubular wall These findings are consistant with the concept that the cells lining the wall of the proximal tubules have the physical character of a highly viscous gel which at least temporarily resist minor pressure changes but which will give way to large changes — and possibly only to large changes — until the pressure inside is equal to that outside the wall This finding indicates that it is physically possible that primary changes in the rate of reabsorption may determine the variations found in the rate of filtration (Leyssac 1963) since in the case the tubular wall would give way immediately to any local tendency in change of the luminal pressure caused by a change in the rate of reabsorption in the single nephron a

change in luminal diameter and not in pressure would be the only result Thus if the rate of proximal reabsorption increases in a nephron the proximal intratubular pressure may decrease to such a degree that a pressure difference of a few mm Hg may exist between the lumen and the surrounding intratubular and peritubular pressures without changes in the luminal diameter The increased rate of reabsorption thereby increases the filtration rate by increasing the effective filtration pressure

In view of these observations the range of proximal intratubular pressures in the normal individual kidneys which vary within the single kidney from one area to the other at a certain time within a range of about 10—18 mm Hg with a mean pressure of about 14 mm Hg is an indication that the intratubular pressure in the single nephron is defined by a control mechanism and pressure differences may exist between single proximal lumina and the surrounding interstitial and intratubular pressures Since however we do not know the changes in the glomerular capillary pressure likely to occur simultaneously with primary changes in the proximal reabsorption an accurate quantitative calculation of the actual changes in the effective filtration pressure is not possible at present As a rough estimation 55 mm Hg may be accepted as a likely glomerular capillary pressure With an average oncotic pressure in the glomerular tufts of about 30 mm Hg the filtration pressure will be 25 mm Hg At a mean pressure of 13—15 mm Hg in the Bowman s space the effective filtration pressure will be 10—12 mm Hg Variations of about 5 mm in this pressure may well account for the physiological variations in the glomerular filtration rate of 30—40 per cent

The present data do not answer the question of why the peritubular capillary pressure increased to a peak following a large dose of angiotensin which constricts the afferent arterioles to such a degree that the filtration pressure is seriously reduced A most likely explanation of the occasional increase in the observed peritubular capillary pressure is a backpressure from an increase in central venous pressure to those capillaries in which the flow is not completely abolished This increase in venous pressure is a nonspecific response to large doses of various endogenous pressor substances including angiotensin (Bock and Meyer 1963)

Since the reabsorption during the occlusion period after interruption of the renal circulation and filtration was found to continue at an unaltered rate until no fluid was left in the proximal lumen (Leyssac 1963) it may be concluded that the demonstrated pressure difference across the tubule wall during that period has not influenced to measurable degrees the rate of transcellular transport

The initial pressure decline in the proximal tubules following interruption of the renal circulation and filtration is simply explained by the continuous reabsorption from the tubules the cellular wall of which either resists for a moment the induced pressure changes without changing the luminal diameter or perhaps even gives way a little because of the abrupt pressure drop to zero in the surrounding peritubular capillaries The change in the slope of the pressure-decline curve demonstrates that a positive hydrostatic pressure is created within the lumen during the occlusion period apparently due to some tension in the tubular wall This tension is most likely physiochemical in nature an interfacial tension between the tubular fluid and the proximal tubular wall like that described by Burton (1962) and which is responsible for the minimal or residual critical closing pressure of 5—10 mm Hg in the vascular system which could be abolished by bile salts and other surface tension lowering agents Since it lasts a few sec until this force or tension manifests itself in the recorded pressure decline curves allowing the proximal intratubular pressure to drop to 5—7 mm Hg

it may be assumed that the internal radius has already been reduced to some extent At a luminal radius of 5 μ (5×10^{-4} cm) the interfacial tension responsible for a pressure of 6—7 mm Hg may be calculated from the equation given for critical closing pressure (CCP)

$$T = CCP \times r$$

An intratubular pressure (corresponding to the critical closing pressure) of 6—7 mm Hg would correspond to an interfacial tension of 4—5 dyn per cm which seems to be a reasonable value compared with surface tension of plasma versus air of 70 dyn per cm

The reason why this phenomenon was not observed in the case of peritubular capillaries is probably that the internal radii of these sinusodial capillaries are not reduced during the occlusion period On the contrary the internal radius increases when the tubular fluid is transposed from the lumen to the interstitial space (compare Fig 4 and Fig 5) Furthermore the peritubular capillaries appear to be linked to the peritubular basement membranes with consequence that the capillary diameters are passively increased when the tubular diameters decrease This may explain why the capillary pressure drops to zero value perhaps even below the renal venous pressure

The observation that the proximal cellular volume was unaffected during the occlusion period indicates that no serious interference with the pump mechanism has occurred unless the passive permeabilities might have decreased pari passu This agrees well with the previous finding of an unchanged rate of transcellular transport during this period but does not support the suggestion made by Longley and Burstone (1963) on the mechanism by which nuclei are discharged into the tubular lumen However as soon as the reabsorption of the luminal fluid was completed a cellular swelling did occur (Fig 6) This effect might be due either to a lack of oxygen and/or to the absence of luminal fluid and/or perhaps even to some still unknown factors the blood The possibility of cellular anoxia as the only factor seems less likely it was observed that proximal cells of kidney cortex slices (0 2—0 3 mm thick less) did not gain their original volume when slices were transferred to oxygenated ...inger solutions or oxygenated plasma immediately after cutting On the contrary further swelling did occur (Bojesen and Leyssac in prep) Irreversible anoxic damage s excluded by the demonstration that the rate of reabsorption was even increased in second occlusion period 2—3 min after re establishment of glomerular filtration and refilling of the tubules following the first registration of occlusion time after in ection of angiotensin (Leyssac 1964 a) It seems important that the observed cellular welling following luminal occlusion may be due not only to lack of oxygen but to lack of luminal fluid The problem why an impaired luminal access to electrolytes mplies a leakage of sodium and water into the cell is not understood from the present knowledge of the transcellular transport mechanism

The present finding that the proximal cellular volume is not significantly changed within 5—10) in the overall range of spontaneous variations of the proximal alt T max (Fig 7) might well fit the concept that the luminal process of uptake or permeability is the limiting point in the rate of transcellular transport in mammalian proximal tubules However the calculated very low intracellular sodium concentrations in functioning proximal tubules (Bojesen and Leyssac in prep) (about 10 meq/l) mplies that the change in intracellular sodium content which might result from an ncrease in the electromotive force of the sodium extrusion pump would be too small cause any change in cellular volume detectable by the present method Thus the

present data do not permit definite answers to the question whether the active sodium pump determines the luminal entry of filtrate or whether a luminal process is rate limiting to the active pump The indication of the importance to the pump of the access of filtrate to the luminal brushborder membrane points to the latter concept as the more likely one

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Acta physiol scand 1965 63 46—54

From the Department of Physiology Australian National University Canberra Australia

Presynaptic Depolarization of Group I Muscle Afferents by Contralateral Afferent Volleys

By

M S Devanandan Birgitta Holmqvist[1] and T Yokota[2]

Received 20 April 1964

Abstract

Devanandan M S B Holmqvist and T Yokota *Presynaptic d polari ation of group I muscle afferents by cont alateral afferent volleys* Acta phys ol scand 1965 *63* 46—54 — Presynaptic depolarization of group I afferents produced by volleys in contralateral hind limb nerves has been investigated. It was found that a train of volleys in contralateral muscle or skin nerves d d ot alter the excitability of the Ia fibres or the size of the monosynaptic EPSP in motoneurones *there is no contralateral presynaptic inhibitory action onto group Ia fibres* A train of s in contralateral muscle and skin nerves increased the excitability of the contralateral fibres to the flexors and extensors The time course of the increased excitability had a latency 10-30 msec a slow rising phase a late maximum at 50—80 msec and a duration of about 150 msec The crossed presynaptic actions originated from high threshold (group II and III) muscle afferent and cutaneous fibres The results also indicate that Ib fibres may have a depolarizing action onto the contralateral Ib nerve terminals

The presynaptic inhibition exerted by ipsilateral afferent volleys onto the group I muscle afferent fibres is due to a depolarization of the afferent nerve terminals (Eccles Magni and Willis 1962 Eccles Schmidt and Willis 1963a) The present investigation is concerned with depolarization of group I muscle afferent fibres produced by contralateral muscle afferent and cutaneous volleys The techniques employed were similar to those in the studies of the ipsilateral actions Dorsal root potentials and increased excitability of the group Ib muscle afferents have been constant findings and it was concluded that depolarization of contralateral group Ib nerve terminals was demonstrated

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[2] Rockefeller Fellow

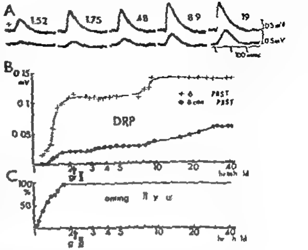

Fig 1 Ipsilateral (+) and contralateral (c) DRPs evoked by 6 volleys (250/sec) applied to the PBST nerve A shows the simultaneous recording from one ipsilateral (upper traces) and one contralateral (lower traces) dorsal root filament at the low L6 segmental level The figures in each record indicate the stimulus strength expressed as a multiple of strength relative to threshold for the nerve A shows a selection of records from which the graph in B is derived where the height of the DRPs (ordinate) is plotted against the stimulus strength (logarithmically scaled abscissa) C shows on the same abscissal scale the size of the first incoming volley (ordinate) Immediately after these series were taken the dorsal roots were cut centrally and the incoming volleys were recorded monophasically Secondary deflections in these monophasic records were taken as a sign of activity in group II fibres and the minimum strength giving this effect are indicated by arrows

Methods

These experiments were performed on spinal (L1) cats which were lightly anesthetized with pentobarbital sodium. The ventral roots L5—S1 were cut on the testing side

Two techniques were mainly employed in testing presynaptic depolarization 1) the recording of the depolarization electrotonically spread to dorsal root filaments (dorsal root potentials DRP) and 2) the measurement of electrical excitability of the group I afferent nerve terminals (cf Eccles 1963 1964) Inhibition on the Ia pathway was also investigated by the effect on the monosynaptic EPSP The experimental procedures resemble closely those previously employed in this laboratory and full descriptions are given in earlier papers

A special effort was made to relate the evoked actions from muscle nerves to their various sub-groups (the Ia Ib II and III group fibre systems) For this purpose the size of the incoming volley in the dorsal root entry zone was separately measured with the conventional double volley technique (Bradley and Eccles 1953 Eccles Eccles and Lundberg 1957) In some experiments appropriate dorsal roots were immediately cut after the crossed actions were investigated and were mounted on recording electrodes so that the maximal group I strength and the threshold of the group II fibres could be determined The late deflection in the monophasic record from the dorsal roots was taken as an indication of the activity in group II fibres

For stimulating and recording the following nerves have been dissected and these abbreviations given will be used quadriceps (Q) the whole hamstring (HS) or its components separately viz posterior biceps-semitendinosus (PBST) and semimembranosus-anterior biceps (SMAB) gastrocnemius-soleus (GS) flexor digitorum longus and hallucis longus (FDHL) plantaris (PL) extensor digitorum longus and tibialis anterior (DP) superficial peroneal (SP) and sural (Sur)

Results

Dorsal root potentials (DRPs) evoked by contralateral volleys

A simple method of determining if group I volleys from muscle depolarize contralateral muscle afferents would be to record from contralateral dorsal root filaments (Barron and Matthews 1938 Bremer and Bonnet 1942 Lloyd and McIntyre 1949 Bernhard

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cases however stimulation of group II fibres was associated with an increase in contralateral DRPs

Changes in excitability of group I muscle afferents by volleys in contralateral nerves

In order to study depolarization of whole group I components of primary afferent fibres produced by contralateral volleys the standard technique for testing excitability change in the primary afferent fibres was applied (Wall *et al* 1956 Wall 1958 Eccles, Magni and Willis 1962) A test stimulus was applied to the intermediate nucleus and the antidromic spike potential was recorded in the muscle nerves. Thus, it was recognized that central terminals of group I fibres of the flexor and extensor muscle afferents were depolarized by afferent volleys from contralateral flexor and extensor muscles as well as skin

The time course of increased excitability of the PBST nerve produced by the contralateral HS volleys is illustrated in Fig 2 For comparison, the effect of the ipsilateral GSPL nerve is also shown in the upper left graph When the contralateral HS nerve was stimulated at the strength of 1 81 times threshold there was a slight increase in excitability of the PBST nerve terminals The monophasic recording from the dorsal root showed that at this strength group II fibres were not stimulated When the stimulus strength was raised up to 3 6 times threshold so that a considerable fraction of group II was also activated there was a considerable increase in excitability Further increase in excitability was produced when the HS nerve was stimulated at the strength of 8 times threshold Throughout these three graphs illustrated the time course of the effects resembles the contralateral presynaptic actions onto the cutaneous fibres (cf Eccles *et al* 1964a b) In general the latency of the contralateral effect (10—30 msec) was longer than for the ipsilateral and the rising phase was slower so that the summit was at about 50—80 msec The total duration was about 150 msec (cf Fig 3 4)

In the cases where a good separation in threshold and conduction velocity between Ia and Ib fibres of the HS nerve was obtained stimulation restricted to the Ia component was ineffective in increasing the excitability of the contralateral group I afferents Sometimes an effect was evoked at the strength of group Ib and a progressive increase of excitability was observed in parallel with increased activation of the contralateral group Ib fibres. In other cases however a significant increase in excitability was not observed until the stimulus strength was raised above the maximal group I and sometimes maximal group II strength When employing PBST or SMAB volleys separately for conditioning there were always smaller actions than when stimulated together SMAB often had a negligible action.

The action of the contralateral Q nerve onto the PL-FDHL is shown in Fig 3 Fig 5 illustrates actions of contralateral flexors (PBST) onto extensors (SMAB) and of contralateral extensors (SMAB and GSPL) onto extensors (SMAB) As in the case of the HS nerve the effects of these nerves did not appear until the stimulus strength was increased so that activation of more than half of group I fibres was achieved

The contralateral cutaneous nerves also increased the excitability of muscle afferents In Fig 3C and E the time courses are plotted for the actions evoked by a train of volleys in the Sur nerve onto SMAB and PBST afferents It was noticed that quite weak stimuli were capable of influencing the contralateral group I fibres (Fig 3D) which indicates that the lowest threshold cutaneous nerve fibres mediate these effects as well as higher threshold ones. No significant difference was observed if other cutaneous nerves were used for conditioning

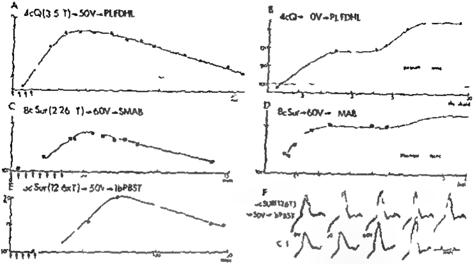

Fig 3 Action of contralateral Q and Sur nerves onto group I afferent nerve terminals Time course and stimulus strength series for increased excitability of the group I fibres of the PL-FDHL nerve are plotted in A and B four volleys being applied to the contralateral Q nerve Excitability changes in the group I SMAB nerve terminals evoked by 8 conditioning volleys to the contralateral Sur nerve are plotted in C and D E shows effects of 5 conditioning volleys applied to the contralateral Sur nerve onto the Ib PBST nerve fibres specimen records of which are illustrated in F The antidromic spike in F was that of Ib fibres of the PBST nerve

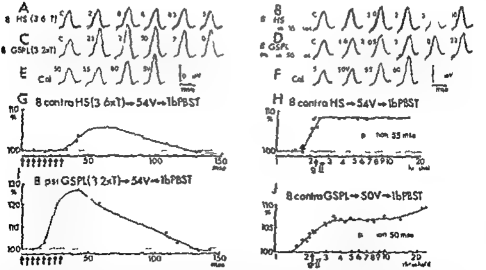

Fig 4 Time courses (G and I) and strength series (H and J) for increased excitability in the Ib PBST fibres when 8 volleys were applied to contralateral HS (G H) GS (J) and ipsilateral GS (I) nerves Samples of records from which the graphs are plotted are illustrated above Arrows in H and J indicate threshold values for group II fibres estimated from the late deflection in the monophasic recording of the afferent nerve discharges

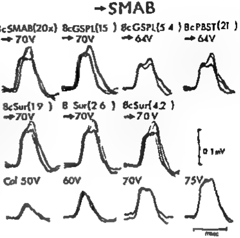

Fig 5 Excitability changes in the SMAB central nerve terminals when 8 conditioning stimuli were applied to various contralateral nerves at stimulus strengths indicated. Each record in the upper two rows consists of 4 expanded traces 2 traces show the unconditioned discharge evoked by a pulse applied in the intermediate nucleus the other 2 superimposed traces (always greater) show the discharge when conditioned by contralateral nerves as indicated above each record The lowermost row shows the increase of the unconditioned discharge at various strengths of cond stimulus i e calibration series

Crossed presynaptic action onto Ia fibres

It is well known that after entrance into the spinal cord the Ia fibres on the ipsilateral side terminate in the intermediate nucleus as well as in the motor nucleus while the Ib fibres do not reach the motor nucleus This anatomical arrangement has been employed to test excitability of Ia fibres selectively by applying a square pulse to the region of the motor nucleus Thus it was found that contralateral volleys from muscle and skin nerves (HS DP GS Pl Sur and SP) had no detectable effect on the excitability of Ia fibres in contrast with the considerable increase (usually more than 130 %) evoked by ipsilateral muscle volleys (PBST)

Furthermore in the 9 flexor (DP and PBST) and 20 extensor (PL-FDHL and GS) motoneurones investigated the monosynaptic EPSP was not significantly depressed by contralateral muscle or skin volleys (HS DP GS PL-FDHL Sur and SP) while considerable depression was evoked by ipsilateral muscle afferent volleys (cf Frank and Fuortes 1957 Frank 1959 Eccles Eccles and Magni 1961)

Crossed presynaptic actions onto Ib fibres

The above noted results suggest that the contralateral presynaptic actions onto group I fibres described earlier are in fact only acting onto the Ib component In order to examine this postulate the excitability changes in the central terminals of Ib fibres were selectively tested by the technique of collision blockage (Eccles *et al* 1963a) Fig 4 illustrates the actions so demonstrated on the excitability of the Ib PBST fibres by volleys in contralateral HS (A B G H) contralateral GSPL (D J) and ipsilateral GSPL (C, I) It is noticeable that the time course (Fig 4G) as well as the effects of increased stimulus strength (Fig 4H and J) matched fairly well the results obtained by testing the excitability of the whole group I component (cf Fig 2 which is from the same experiment as Fig 4) hence it is most likely that the crossed action onto the whole group I component is contributed by its Ib component

In Fig 5 another method has been used to demonstrate that extensor Ib fibres receive crossed actions Stimulation of the central nerve terminals in the intermediate

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From the Aero-Medical Laboratory Institute of Physiology University of Lund Sweden

Mixed Venous CO_2 Tension — Determined by a CO Rebreathing Method Influence of Rebreathing Time

By

G LUNDIN and D THOMSON

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Abstract

Lundin G and Thomson D *Mixed venous CO tension — determ ed by a CO rebreathing method I fluence of rebreathing time* Acta physiol scand 1965 *63* 55—57 — The influence of rebreathing time when using a CO rebreathing method for graphical determination of the mixed venous CO tens on was stud ed An almost significant difference of 0 6 mm was found between values obtained at reb eathing rates of 30 and 50 breaths per min with rebreathing times of [illegible] and 13 sec This should give a difference at rest of about 5 per cent when the card ac output is calculated with the higher value when the fast rate is used fo the mixed venous CO deter min t on The solub l ty of CO in lung tissue probably doses not cause this difference

In a previous paper — Jernérus Lundin and Thomson (1963) — a modification is described of Defares (1956) indirect method of determining the mixed venous CO_2 content and from that the cardiac output The assumption was made that the arterial blood has the same CO_2 tension as that of the end tidal CO_2 The method involves rebreathing at a constant frequency as a means of determining the mixed venous CO_2 and the use of a rapid infrared CO_2 meter for continuous recording of the CO_2 concentration of the rebreathed gas and of the end-tidal air Given the CO production and the a v CO_2 difference the cardiac output can be calculated by Fick s equation However in a recent handbook of physiology Hamilton (1962) states that owing to the combination of CO_2 with the fluids of the lung tissue it is impossible to use the lungs as a tonometer for determining the mixed venous CO_2 tension Hamilton claims that there is a constant increase in the values obtained for mixed venous CO_2 tension as rebreathing time is prolonged this being caused at first by slow mixing slow diffusion and slow equilibrium with arterialized fluid in the lungs and later by recirculation of blood which has been exposed to rebreathed pulmonary air This gives an increased a v CO_2 pressure difference and thus with long rebreathing times cardiac output would be calculated as low and with short rebreathing times the figure would be high We found it important to investigate whether this also holds good for the rebreathing method used by Jernérus *et al* (1963)

TABLE I Oxygenated mixed venous blood PCO_2 mm Hg

Subj	No of expts	Freq 30/min Mean	Range	Freq 50/min Mean	Range	Difference mm Hg Mean	Range
L L	10	52.8	50.6—55.2	51.6	48.5—54.7	+1.2	−1.4—+3.9
R L	10	48.2	41.7—52.7	47.5	42.8—52.7	+0.6	−4.2—+3.8
U W	10	51.2	49.2—53.7	51.3	49.9—53.7	−0.2	−2.8—+2.[illegible]
G L	10	51.2	47.8—55.0	50.6	43.6—54.6	+0.6	−2.5—+4.2
D T	10	49.2	45.0—51.4	48.8	45.0—51.3	+0.5	−1.3—+2.4
L Å	10	53.0	51.1—56.4	52.1	48.8—56.4	+0.9	−1.4—+4.4
Mean	60	50.9	41.7—56.4	50.3	42.8—56.4	−0.6	−4.2—+4.4

Method

The CO_2 tension of the mixed venous blood is determined by rebreathing in a bag which at the beginning contains about 1 litre of a gas mixture with a composition of about 2 % CO_2, 40 % O_2 and 58 % N_2. The subjects breath in and out of the bag, the breathing rate being governed by a metronome. The CO_2 content of the gas in the bag is determined breath by breath by means of a rapid infrared CO_2 meter (Godart). The CO_2 tension in the bag increases gradually in an exponential way with the number of respirations. The values for CO_2 tension measured during 11 rebreathings from the bag can be used for a graphical determination of the CO_2 tension of the mixed venous oxygenated blood. For details see Jernerus, Lundin and Thomson (1963). Six normal subjects were used. The rebreathing rates from the bag were 30 and 50 inhalations per min, and rebreathing times 22 and 13 sec, respectively. Ten determinations at each rate were done on each subject. Between each rebreathing there was an interval of about 10 min. The experiments were arranged two by two, starting with the slow and fast rate alternately. The subjects were allowed to rest for about half an hour before the start but were not in a basal state.

Results

The results of 60 expts are summarized in Table I. The average mixed venous CO_2 pressure is 50.9 mm Hg with a rebreathing rate of 30 breaths per min and 50.3 mm Hg with 50 per min. *Thus a difference in mixed venous CO_2 pressure of 0.6 mm determined with the two rebreathing rates was found.* Analysis of variance has been made on the original values of observation with regard to possible differences between subjects, between experiments within subjects and interaction between subjects and methods. The obtained difference of 0.6 mm was shown to be almost significant ($0.01 < P < 0.02$).

Discussion

The difference obtained between the mixed venous CO_2 pressure determined with the 2 breathing rates should give a smaller value for cardiac output with the slower re-

breathing rate This difference if we assume a CO_2 pressure in arterial blood of 38 mm should thus give a relationship between the a v differences of

$$\frac{50\,9-38}{50\,3-38} = 1\,049$$

Assuming the CO_2 dissociation curve to be a straight line in this pressure region we obtain about [illegible] per cent higher values for cardiac output determined with the fast breathing rate in comparison with the values found with the slow rate This difference is considerably smaller than the values given by HAMILTON who claims an a v difference 17 per cent higher with a rebreathing period of 24 sec compared with one of 16 sec The explanation is probably that the solubility factor does not disturb the exponential course of the increase in CO in the lung bag system and thus does not influence the values of mixed venous CO_2 to the same extent as in the plateau CO_2 methods The small difference between the values can however be explained by other factors One is the recirculation of blood which has passed through the lung during rebreathing In our experiments however the CO_2 percentages of the first 3 or 4 rebreathings are below the end tidal CO_2 value which means that the first recirculated blood if any should have a depressing influence on the course of the CO_2 accumulation in the bag and induce smaller mixed venous blood CO_2 values in the calculations The time for rebreathing after the lung bag mixture has reached the end tidal CO value is with both breathing rates too small to allow any significant recirculation of blood which has passed through the lung when their alveolar CO_2 pressure has been higher than during normal breathing The buffering action of the tissues due to their ability to dissolve CO_2 and of the residual blood in the left heart should also reduce any recirculation effect Another factor which in our method may have an effect on the mixed venous CO_2 values obtained could be the volumes changes caused by a gradually decreased CO_2 elimination and a constant O_2 uptake a factor which should be more pronounced with a slow rebreathing rate This last factor could explain the rather small difference observed between the CO_2 pressure values found with the two breathing rates The somewhat large differences between intraindividual values in mixed venous CO pressure partly depends on the fact that the subjects were not always in a basal state and partly on the error of the method

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JERNÉRUS [illegible] G LUNDIN and [illegible] THOMSON Cardiac output in healthy subjects determined with a CO rebreathing method *Acta physiol scand* 1963 *59* 390—399

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Peripheral Resistance Response to Occlusion of Visceral Arteries

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Abstract

Nissen O I *Peripheral resistance response to occlusion of visceral arteries* Acta physiol scand 1965 63 58—67 — In order to compare the effect of visceral and carotid sinus hypotension on the resistance vessels the peripheral resistance in 3 areas (hind body kidneys and head) in the cat has been investigated by means of the constant perfusion rate technique during occlusion and desocclusion of visceral (coeliac superior mesenteric and renal) arteries and of the carotid arteries In all 3 areas clamping of the visceral arteries provoked a vasodilatation whereas a vasoconstriction in the hind body and kidney was observed during carotid artery occlusion The clampings always produced significant rises in the general arterial blood pressure The pressor response caused by visceral artery occlusion thus is produced in spite of a widespread dilatation of the resistance vessels — not as a consequence of a constriction in the same vessels Denervation of the carotid sinuses plus section of the vago sympathetic trunks nearly always abolished the vasodilatation Stabilising the blood pressure by means of a compensating device always abolished the vasodilatation during visceral artery occlusion Under these circumstances the vasodilatation was not replaced by a vasoconstriction in the periphery It has thus not been possible to demonstrate baroreceptor activity in the visceral arteries even under circumstances where the activity of the sino-cardio-aortic baroreceptor reflexes was eliminated

The existence of baroreceptors in the visceral arteries and their possible importance in the regulation of the systemic arterial blood pressure has been under dispute since the work of Gammon and Bronk from 1935 The present work is a contribution to this dispute

The systemic arterial blood pressure is the resultant of the peripheral hemodynamic resistance and the function of the heart therefore the present author has chosen to record the changes in these basic parameters following visceral artery occlusion and to use them in an evaluation of the possible significance of visceral artery baroreceptors

The changes in the former parameter are dealt with in this paper while the subject of a subsequent paper will be the changes in the latter and some observations on factors determining the magnitude of the blood pressure rise following occlusion of the mesenteric arteries

Methods

The experiments were performed in cats anesthetized with 60 mg chloralose and 210 mg urethan per kg i p supplementary doses were rarely required In 2 expts (kidney perfusions) the cat was decerebrated in ether narcosis at least 2 hours prior to the occlusion tests All experiments were performed with the cat in the supine position on a heated table the top of which was used for zero setting of the manometers as it was almost at level with the right atrium A tracheal cannula was inserted routinely

The vagosympathetic trunks were exposed to make them ready for cutting later in the experiment Since the depressor nerve in the cat runs very close to the vagosympathetic trunk cutting the latter generally also involves cutting the depressor

In some experiments also the carotid sinuses were dissected free ready for denervation The denervation was performed by clearing the common and external carotid arteries for nerve fibres at their junction plus excision of the first part of the internal carotid artery with attached nerve fibres

The arterial blood pressure was recorded by means of a mercury manometer connected with the right brachial artery in a few cases with a carotid artery Silk ligatures were placed loosely around the coeliac and superior mesenteric artery around both carotid arteries and in some cases around the renal arteries The ligatures were passed through polyethylene tubings the ends of which were slightly narrowed by flaring This was done to keep the vessel from being drawn up in the tubing when occlusion was performed by pulling the thread On placing the ligatures injury to the nerve fibres was avoided as far as possible

The occlusions were maintained until maximal response was obtained generally 1/2 to 2 min

To elucidate the variation of the peripheral resistance during occlusion of the visceral and carotid arteries 3 large areas have been perfused using the constant perfusion rate technique with arterial blood from the cat itself The perfusion pressure was recorded with a mercury manometer connected with a T cannula inserted on the perfusion tubing just in front of the artery cannula the lumen of which was chosen as large as possible the pressure fall in this cannula has been about 5 mm Hg — varying with the blood flow No corrections for this pressure fall have been applied In one experiment the pressure in the superior caval vein was recorded with a water manometer via the right jugular vein All pressures were recorded on a smoked drum

The pump used for perfusion was of the membrane type the movements of the membrane being produced by a waterfilled 10 ml syringe the stroke length of which could be varied continuously during the experiments At both the inflow and outflow of the pump-chamber the blood passed windkessels consisting of a soft rubber tubing and a small partly airfilled glass reservoir respectively Both were chosen so small that changes in the extracorporeal blood volume during variations in the inflow and exit pressure amounted to only a few ml In the small glass reservoir a coarse wire gauze filter was inserted to catch the few blood clots formed in spite of the heparinisation Between the outflow of this reservoir and the inflow in the artery the blood passed a glass spiral immersed in water at 38—39 The piston delivered 48 strokes per minute and this frequency multiplied by the stroke volume gave an approximate measure of the perfusion rate Since the pump was provided with simple rubber inlet and exit valves the pressure on the inflow side had to be kept below the outflow pressure However even when this condition was fulfilled the minute volume of the pump varied a little with both the input pressure and the output pressure (at the most plus or minus 6 per cent with a change in input or output pressure of 50 mm Hg) The former source of error was avoided by keeping the input pressure (read on a mercury manometer) constant by way of a clamp on the polyethylene tubing conducting the blood from the [illegible] to the pump

Before the experiment started the pump-chamber with connecting tubings were filled with saline with careful removal of all air bubbles The cat was heparinized (?0 mg [illegible]) immediately before connection with the pump After the connection donor blood was administered as replacement for the small blood loss during the operation and the bleeding in the pump

In some of the experiments the systemic arterial blood pressure was effectively stabilized by means of a compensating device — a [illegible]00 ml transfusion bottle partly filled with blood — which was connected with the arterial system of the animal The air pressure in the bottle could be adjusted to the selected blood pressure level

Hind body perfusion (7 expts) The pump was inserted on the abdominal aorta just proximal to the common iliac [illegible] The inferior mesenteric artery was generally ligated and at the

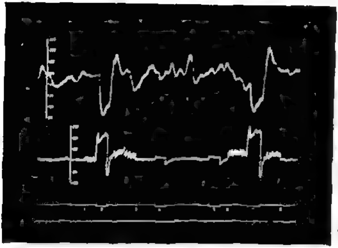

Fig 1 Cat weight 5 6 kg hind body perfusion *The vagosympathetic trunks are cut* the carotid sinuses are not denervated Upper curve *Perfusion pressure lower* curve Systemic blood pressure Time marking every 6 second in this and the following figures

1 the pressure bottle is disconnected 2 occlusion *of the coeliac and superior* mesenteric arteries the bottle is connected at 3 4 and 5 combined occlusions of the coeliac and superior mesenteric arteries 6 the pressure bottle is disconnected *7 occlusion of the coeliac* and superior mesenteric arteries 8 the pressure bottle is connected

level with the divided aorta a tight ligature was placed around the large intestine and all *visible anastomoses between the perfused area and the mesenteric vessels were ligated In one* experiment such an anastomosis (arterial arcade) was open and in another the inferior mesenteric artery was not ligated the results from these experiments did not differ from the remainder

In 2 of the 7 expts the blood pressure compensating device was used (In these all visible anastomoses between the mesenteric arteries and the perfused area were ligated) The blood in the bottle was connected with the blood stream flowing from the central end of the divided aorta towards the pump by means of a polyethylene tubing and a T cannula

Kidney perfusion (6 expts) The pump was supplied with blood *from the left common carotid* artery and the output delivered to the central part of the abdominal aorta divided below the renal arteries The operation was performed through a lumbar approach on the left side which made it easier to ligate the aorta above the renal arteries without damaging the splanchnic and renal nerves The lumbar arteries leaving the perfused part of the aorta were ligated

The hind body lacked its natural blood supply in this type of experiment

Of the 6 kidney perfusion expts 3 were performed with the compensating bottle connected with either the superior mesenteric artery or a large branch of the coeliac artery In the former case occlusions were made on the coeliac artery in the latter on the superior mesenteric artery Two of these experiments were performed on decerebrated cats

Perfusion of the head (2 expts) Inflow to the pump from the central part of a divided common carotid artery outflow in the peripheral parts of the divided common carotid arteries *The* vertebral arteries were not ligated

In the present work the perfusion pressure is used as an expression of the peripheral resistance in the area perfused with the constant output pump This is permissible if the following conditions are fulfilled

1 The venous pressure must be near zero and — more important — it must not alter significantly *during the occlusions If this should not be the case it would be necessary to use the difference* between perfusion pressure and venous pressure as an expression of the resistance. However the cava vein pressure is low and it does not alter significantly during the occlusion (cf results) both conditions may thus be considered fulfilled

2 Arterial anastomoses must not exist between the area perfused by the pump and the area perfused from the arteries which are clamped In the presence of such anastomoses the pressure difference between the two areas suddenly increased by the occlusion would increase the flow through the anastomoses and this would tend to lower the perfusion pressure and thus simulate vasodilatation In the present experiments this source of error has been eliminated

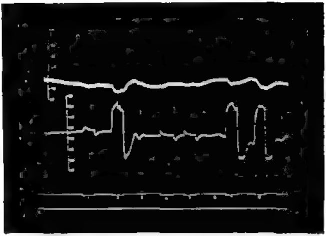

Fig 2a Cat weight 2 9 kg Hind body perfusion *The carotid sinuses are denervated* the vagosympathetic trunks are intact. Upper curve Perfusion pressure lower curve Systemic pressure

With the pressure bottle disconnected 1 occlusion of the carotid arteries (denervation control) 2 occlusion of the coeliac and superior mesenteric arteries 3 pressure bottle is connected 4 and 5 combined occlusions of the coeliac and superior mesenteric arteries 6 pressure bottle is disconnected 7 and 8 combined occlusions of the coeliac and superior mesenteric arteries

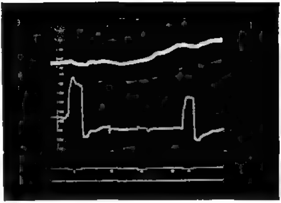

Fig 2b Same preparation but the *vagosympathetic trunks are cut* The *sinuses are denervated* Upper curve Perfusion pressure lower curve Systemic pressure

With the pressure bottle disconnected 9 occlusion of the coeliac and superior mesenteric arteries 10 pressure bottle is connected 11 occlusion of the coeliac and superior mesenteric arteries 12 the pressure bottle is disconnected 13 occlusion of the coeliac and superior mesenteric arteries

by ligating the actual anastomoses (arterial arcades along the intestine and the inferior mesenteric artery in the hind body perfusion experiments)

3 Significant arterial anastomoses between the perfused area and the systemic circulation must be absent if they were present a systemic pressure rise may simulate a vasoconstriction in the perfused area or neutralize or reduce the effect of a vasodilatation in the area on the perfusion pressure In hind body and kidney perfusions these anastomoses are of no quantitative significance (besides if the systemic pressure is stabilized this source of error is also theoretically eliminated) In the head perfusions on the other hand this flow through anastomoses between the perfused area and the systemic circulation may be of a considerable magnitude (vertebral arteries spinal arteries) and this may have caused erroneously small falls in the perfusion pressure during clamping of the visceral arteries in these experiments The two-phased course of the perfusion pressure curve observed in these cases (*cf* results) suggests that errors have been introduced by this mechanism the first phase of rising perfusion pressure possibly being a result of the rise in systemic pressure transmitted through anastomoses to the perfused area

That the resistance changes in the perfused areas should be hormonally elicited is excluded because the reactions appeared a few seconds after the systemic pressure rise i e in a period much shorter than the mean circulation time through the pump

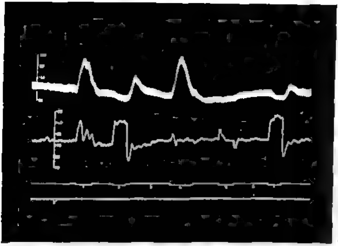

Fig 3a Cat weight 4 9 kg Kidney perfusion The carotid sinuses are intact the vagi are intact Upper curve Perfusion pressure lower curve Systemic pressure

With the pressure bottle disconnected 1 occlusion of the carotid artery 2 occlusion of the coeliac artery 3 the pressure bottle is connected 4 occlusion of the carotid artery 5 occlusion of the coeliac artery 6 the pressure bottle is disconnected 7 occlusion of the coeliac artery

Fig 3 b Same preparation but the *carotid sinuses are denervated* The *vago sympathetic trunks are cut* Upper curve Perfusion pressure lower curve Systemic pressure

With the pressure bottle connected 1 occlusion of the coeliac artery 2 occlusion of the carotid artery 3 the pressure bottle is disconnected 4 occlusion of the coeliac artery 5 occlusion of the carotid artery 6 occlusion of the coeliac artery 7 the pressure bottle is connected 8 and 9 occlusion of the coeliac artery

It is reasonable to accept the resistance of the 3 perfused areas as representative for the total peripheral resistance of the body as nearly all sorts of tissues supplied by the greater circulation are represented in natural proportions in the 3 pump perfused areas

Results

The *typical reaction* to visceral artery occlusion may be seen from Fig 1 no 2 and 7 Combined clamping of the coeliac and superior mesenteric arteries provokes a significant hypertensor effect in the systemic pressure (lower curve) however this pressure rise is associated with a marked fall of the perfusion pressure (upper curve) If the systemic blood pressure was stabilized by means of the compensating device (Fig 1 no 4 and 5) clamping of the visceral arteries did not provoke any change in the peripheral resistance (this fact is also illustrated in Fig 2 and 3) Likewise if all the classical

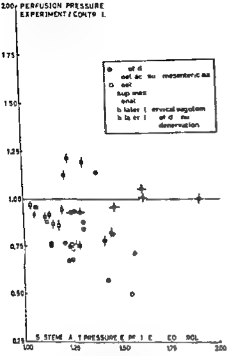

Fig 4

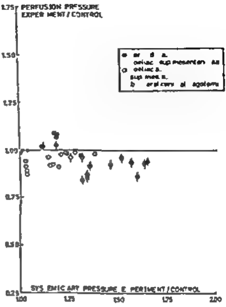

Fig 5

Fig 4 Hind body perfusions Responses to occlusions performed on 5 cats

Fig 5 Kidney perfusions Responses to occlusions performed on 3 cats

baroreceptors were denervated (Fig 2 b and Fig 3 b) occlusion of the visceral arteries never produced significant alterations in the perfusion pressure

From Fig 2a no 2 7 and 8 it may be seen that if the cardio-aortic regions alone are intact (carotid sinuses denervated) a vasodilatation in the resistance vessels during visceral artery occlusion is still produced

The typical reaction to carotid artery occlusion (Fig 3 a no 1) consists of a rise as well in the systemic pressure (lower curve) as in the perfusion pressure (upper curve) as one might expect the latter rise is not abolished if the blood pressure is stabilized (Fig 3 a no 4) Carotid sinus denervation abolished the rise (Fig 3 b no 2 and 5 Fig 2 a no 1)

The results of all the occlusions are given in the following

Hind body perfusions Fig 4 shows the fractional changes in perfusion pressure (ordinate) in relation to the corresponding changes in systemic artery pressure (abscissa) when different visceral arteries were occluded The systemic control pressures were above 100 mm Hg at all occlusions apart from 3 where they amounted to 97 80 and 91 mm Hg The results represent only the 5 expts in which the compensating device was not used

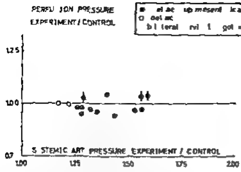

Fig 6 Head perfu ions Responses to occlusions performed on 2 cats

The results of [illegible] occlusions of the renal arteries are omitted in Fig 4 because of defects in the technique of occlusion Due to insufficient narrowing of the ends of the polyethylene catheters through which the silk loops around the renal arteries were passed the arteries were drawn into the catheters almost to their full length during the occlusions consequently these occlusions produced intense rhytmical oscillations in the perfusion pressure in 2 cases minor to medium rises (in the same pressure) in 2 cases and no changes in 2 cases The reactions have been interpreted as the answer to stretch and irritation of the peritoneum the surrounding nervous tissue and perhaps the adrenals

Kidney perfusions The results of the 3 expts in which the pressure bottle was not used are summarized in Fig 5 in the same way as for the hind body perfusions The systemic control pressures were above 100 mm Hg except at 2 occlusions (73 and 97 mm Hg) Fundamentally the results are the same A rise in perfusion pressure when clamping the carotid arteries and a fall in the same pressure during occlusion of the visceral teries The variations in the peripheral resistance are not as marked as in hind body perfusions

In 1 clamping of the superior mesenteric artery a rise in the perfusion pressure was noted and it appeared that a few nerve twigs were included in the silk loop around the artery and were in this way mechanically irritated during the occlusion Consequently this result is not included in Fig 5

In 3 expts the pressure bottle was used the course of 1 of them is demonstrated in Fig 3 2 of these experiments were performed on decerebrated cats

Head perfusions The results from 2 expts are given in Fig [illegible] At 6 of the occlusions the control pressures were below 100 mm Hg (88 86 94 97 95 and 95 mm Hg) The fall in the perfusion pressure upon clamping the visceral arteries was not great Most frequently a small quick rise appeared at first followed in the next few seconds by a fall below the control pressure so that the net change appeared to be negative

Cutting both vagi (without cutting the sympathetic trunks) altered the perfusion pressure response so that only the first quick rise was present An identical result was observed if only the sympathetic trunks were cut

Discussion

Gammon and Bronk (1935) observed a nervous discharge from the Pacinian corpuscles in the mesentery of the cat resulting from an increase of pressure in the mesenteric arteries No significant change in the systemic arterial pressure could however be

demonstrated when the superior mesenteric artery was perfused with wide variations in the perfusion pressure Nor was Heymans *et al* (1936) able to show — in the spinal dog — any effect in the general blood pressure of decreasing or increasing the pressure in the mesenteric arteries

The opposite view was set forth by Bennati and Gluggino (1936) who found indication of the importance of visceral arterial baroreceptors in the observation that in dogs anesthetized with morphine and chloralose acute occlusion of the large visceral arteries (in particular the coeliac and superior mesenteric arteries) provoked a greater rise in the arterial blood pressure than clamping of other arteries such as the femoral artery supposedly receiving a similar fraction of the cardiac output

Sarnoff and Yamada (1959) found an even greater effect of similar occlusions in chloralosed cats and they suggested — with some hesitation — that in the cat the mesenteric receptor system has a greater reflex influence on the arterial pressure than that exerted by the carotid sinus and aortic arch receptors

Since this work the problem has been reexamined by several investigators without definitive agreement on the significance of these postulated reflexes most authors however deny their existence and interpret the rise in arterial blood pressure as simply due to the increase in total peripheral resistance produced by the occlusion and to a redistribution of some of the blood previously contained in the splanchnic area

An approach to the problem lies in the isolated perfusion of the hypothetic receptor area allowing local alteration of the arterial pressure to be made without direct mechanical interference with the rest of the circulation This method which has had such great success in the studies on the carotid sinus is technically very difficult in the case of the coeliac and mesenteric arteries since damage to the local nerves can hardly be avoided Yet Yamada (1962) using this method in dogs claimed that an increase in mesenteric pressure produced a fall in the systemic pressure From this observation and from occlusion experiments similar to those performed by Sarnoff and Yamada in cats he accepted the conclusion of the latter authors on the importance of baroreceptors in the area perfused by the coeliac and mesenteric arteries and stated that the increase in the systemic arterial pressure observed after occlusion of these visceral arteries in animals with denervated aortic arch and carotid sinuses were due to a reflex elicited from these receptors The perfusion method had — as mentioned above — been used by Gammon and Bronk without positive evidence of mesenteric baroreceptors

The present experiments show that occlusion of the abdominal arteries in the presence of intact sino-cardio-aortic baroreceptor zones produces a vasodilatation in the perfused areas while clamping of the carotid arteries in the presence of the intact hypothetical zones in the visceral arteries yield a vasoconstriction in the perfused area

The possibility that the vasodilatation in the perfused area is reflexly induced from baroreceptors in the visceral arteries is excluded by the fact that the reaction disappears if the sino-aortic areas are denervated or if the central arterial pressure is stabilized by way of the compensating device

Thus the vasodilatation in the perfused area during clamping of the visceral arteries must be a secondary compensatory answer from the sino-cardio-aortic baroreceptors to the blood pressure rise only this interpretation is consonant with the disappearance of the vasodilatation if the blood pressure is stabilized or the classical baroreceptor zones denervated

The experiments show with great certainty that the hypothetical zones in the visceral arteries must — if they exist at all — be of minor importance in comparison with the

known baroreceptors in the sinus and the aortic region as far as their influence on the peripheral resistance is concerned

Even though the hypothetical zones are of minor importance in comparison with the known baroreceptors the possibility existed that a minor reflex activity from the visceral arteries could be uncovered by denervating the classical zones or stabilizing the blood pressure The last method also prevented an increased systemic mean blood pressure from acting on other less well known baroreceptors But even with both methods in use there was no sign of peripheral vasoconstriction during clamping of the visceral arteries

With the technique used in the present work it has thus proved impossible to demonstrate a reflex arch between the visceral arteries and the rest of the peripheral resistance vessels even under circumstances where the sino cardio-aortic reflexes were eliminated

The vasomotor reflexes described by Heymans et al (1936 and 1937) in the spinal dog and claimed to be of a possible importance for the local distribution of blood in the abdominal viscera thus seem to be of no importance for the renal blood flow in the chloralosed or decerebrated cat (Heymans et al reported splenic dilatation and constriction when increasing and decreasing the perfusion pressure within the coeliac and superior mesenteric arteries)

Onnis Shumacker and Bounous (1963) have studied the effect of a longer lasting visceral artery occlusion on the renal blood flow in the dog They measured the blood flow discontinuously In the first determination after the occlusion — after a few minutes — the flow was increased (and the systemic pressure too because of the occlusion of the arteries) but in the following half an hour the renal blood flow decreased in spite of a steady high blood pressure (cf their Fig 6) Onnis et al further noted that the hypertensor effect of clamping the visceral arteries was not abolished in dogs previously (8 days before) subjected to thoraco lumbar sympathectomy and splanchnicectomy They suggested that the hypertensive reaction is not due to a reflex vasoconstriction but to a change in the distribution of blood flow and a reduction in the effective vascular bed

Lemarchands et al (1963) reported that occlusion of the coeliac and superior mesenteric arteries provoked an increase in the volume of the innervated dog kidney separately perfused from a donor dog the authors were in doubt as to the interpretation of this result According to the present work it is reasonable to interpret the increase as a vasodilatatory reflex elicited from the sino-aortic areas

Neil Redwood and Schweitzer (1949 a) observed that chloralose causes a reduced sensitivity of the vasomotor center to baroreceptor reflexes in the cat but not in the dog (Neil Redwood and Schweitzer 1949 b) It is not a question however of an absolute depression of the baroreceptor reflexes for instance Neil himself together with Ead and Green in a later work (1952) used chloralose urethan anesthesia in experiments in which the baroreceptor sensitivity to the pulse pressure was demonstrated by a reflex response Even though the sino-aortic baroreceptors must have been active in the present investigations it could not be excluded that the hypothetical zones in the visceral arteries could be considerably depressed by the chloralose For this reason 2 kidney perfusions were performed on unanesthetized decerebrated cats The results of these were indistinguishable from the rest of the experiments

It must be pointed out that fall in resistance in the hind body and the head is the net result of resistance changes in several tissues Theoretically therefore the possibility

exists that constriction in the resistance vessels in one sort of tissue is dominated by a vasodilatation in another in the area perfused by the pump during visceral occlusion

Likewise a specific reflex arch from the mesenteric arteries to the capacitance vessels or the heart is not excluded from these experiments (cf however a subsequent paper)

Apart from these limitations the results suggest however that the blood pressure rise caused by visceral artery occlusion is mainly of a non reflectory nature (which will be discussed in a subsequent paper) and they demonstrate at least that the pressure rise is not due to constriction of the peripheral resistance vessels but that it occurs in spite of a dilatation of these vessels

Further it has not been possible to demonstrate even a slight baroreceptor activity from these arteries when the function of the sino-aortic regions were eliminated

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Acta physiol scand 1965 63 68—76

From the Institute of Medical Physiology University of Copenhagen Denmark

Heart Rate and Blood Pressure Response to Occlusion of Visceral Arteries

By

OLE I NISSEN

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Abstract

Nissen O I *Heart rate and blood pressure response to occlusion of visceral arteries* Acta physiol scand 1965 63 68—76 In experiments on cats the heart rate response to occlusion of the visceral and carotid arteries has been investigated Of 69 combined or separate occlusions of the coeliac and superior mesenteric arteries 6 produced a tachycardia in 9 no change in heart rate occurred in the rest a bradycardia occurred Of 32 common carotid artery occlusions 2 produced a bradycardia in 4 no change in heart rate was observed and in the rest a tachycardia was produced The bradycardia during visceral artery occlusion is interpreted as a reflex elicited from the sinoaortic baroreceptors and caused by the rise in blood pressure It is further observed that the pressure increments caused by visceral artery occlusion have a maximum at subnormal systemic pressures (between about 65 and 100 mm Hg) This is contrary to the blood pressure rise caused by carotid artery occlusion which as a rule is greater the greater the systemic pressure in accordance with the threshold concept for the baroreceptors

Therefore at low systemic pressures (below about 110 mm Hg) a higher response to combined occlusion of the coeliac and superior mesenteric arteries than to carotid artery occlusion is the rule the opposite is the case at higher systemic pressures

Since the papers of Bennati and Ghiggino (1956) and Sarnoff and Yamada (1959) have revived the old problem concerning the hypothetical baroreceptors in the area perfused by the coeliac superior mesenteric and the inferior mesenteric arteries several authors have reinvestigated the problem The investigators have — as Sarnoff and Yamada and Bennati and Ghiggino — mainly dealt with the effect on the systemic arterial blood pressure of a local pressure change in the hypothetical baroreceptor region (produced either by occluding the mentioned visceral arteries or by perfusing them at different pressures) the problem has been to establish whether the recorded changes in the systemic pressure are reflexogenic or purely of mechanical nature (for example the effect of imposing a new resistance to a fixed cardiac output)

The present author has tried to analyse the hypertensive effect of occlusion of the visceral arteries and has in a preceding paper (Nissen 1964) reported on the alterations in the peripheral resistance provoked by such occlusions These experiments showed in an unequivocal way that the pressure increments are not caused by peripheral vasoconstriction — on the contrary they occur in spite of a widespread peripheral vasodilatation

The possibility still remained open however that the pressure increments might be — at least in part — of reflex nature namely the results of a reflex activation of the

heart. That such a mechanism might be involved is suggested by Sarnoff and Yamada's report on the occurrence of a tachycardia during visceral artery occlusion. Since the combination of peripheral vasodilatation and increased heart rate would be an unusual dissociated effect of the sympathetic system on the circulation the changes in heart rate during visceral artery occlusion were reinvestigated.

Further some observations on the arterial pressure increment caused by visceral (and carotid) artery occlusion as a function of the arterial control pressure (immediately before the pressure rise) will be included.

Methods

The experiments have been performed on 13 cats. 8 were anesthetized lightly with pentobarbital (40 mg/kg i.p. initially plus supplementary doses i.v. later in the experiments), the others with chloralose (60—100 mg/kg i.p.) or chloralose plus urethan (60 mg and 210 mg/kg i.p. respectively).

The trachea was cannulated and the vagosympathetic trunks were exposed for cutting later in the experiments. In the majority of the experiments (10) the blood pressure was recorded by connecting the right femoral artery via a T cannula with two Statham transducers, one (a mechanically damped AA 23 transducer) recording the mean pressure, the other (an undamped Dd 23 transducer) the pulse pressure. The pressures were taken down by an Ultralette light recorder. The heart rate was counted from the pulse curve while the blood pressure was read from the mean pressure curve. In the rest of the experiments the pressure was recorded from a brachial or a carotid artery with a mercury manometer writing on a smoked drum. In these cases the heart rate was counted from an electrocardiogram or directly from the pressure curve on the smoked paper (quick rotating drum).

Through an abdominal midline incision silk ligatures were placed loosely around the coeliac and superior mesenteric arteries and in a few experiments around the abdominal aorta below the renal arteries. Each ligature was passed through a polyethylene tubing the end of which was slightly narrowed by flaring. This narrowing was made to prevent the arteries from being drawn up in the catheters when the occludings were performed by pulling the threads. Injury to the nerve fibres was avoided as well as possible and caution was exerted not to enclose any nerve fibres in the ligature (otherwise these nerve fibres could be mechanically irritated during the occlusions).

Occlusions of one or both of the common carotid arteries with bulldog clamps were made in order to compare the results with those of the visceral artery occlusions. In some experiments one carotid artery was used for blood pressure recording or to establish connection with a compensating bottle. In these cases occlusions were made only on the other carotid artery.

In 6 experiments a blood pressure compensating bottle (described in the previous work (Nissen 19[illegible])) was connected to the left carotid artery in order to investigate the heart rate response to the occlusions when the systemic pressure was stabilized. In some of the experiments the bottle was used to obtain different levels of arterial blood pressure by controlled bleeding and reinfusion.

The occlusions were maintained until a maximal and stable response was obtained, generally for 1/2 to 1 1/2 min. The blood pressure in the period was read in the stable part of the curve. The control pressure was the blood pressure immediately before the occlusion period. The heart rates shown in the figures were generally obtained for a 1/2 min period immediately before the occlusion and for similar periods during the plateau of the pressure rise. (Checks on the rate in other parts of the occlusion periods never disclosed changes in heart rate deviating in direction from those observed during the plateau.)

In 3 out of the 13 expts (2 in chloralose, 1 in pentobarbital anesthesia) the heart rate was not determined in order to concentrate on the pressure increment as a function of the systemic control pressure decreased or increased by controlled bleeding and reinfusion.

Results

Changes in heart rate. The results of the occlusions in 4 pentobarbital anesthetized cats were as follows. Clamping of the carotid arteries provoked a slight tachycardia while

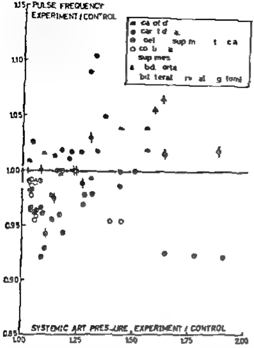

Fig 1 Results of occlusions in 5 chloralose or chloralose urethan anesthetized cats

occlusion of the coeliac and superior mesenteric arteries generally produced a slight bradycardia In 2 out of 7 combined occlusions of the coeliac and superior mesenteric ies performed in 1 expt and in 1 out of 5 similar occlusions performed in another tachycardia was produced

Two occlusions of a carotid artery were performed with the blood pressure effectively stabilized by means of the compensating bottle in these a tachycardia appeared (2 9 and 6 3 per cent of the control rates) In 4 similar occlusions of the visceral arteries a bradycardia was observed in 2 (1 5 and 1 0 per cent) in 1 a tachycardia (0 5 per cent) and in 1 no change was observed

Fig 1 shows the fractional changes in heart rate against the fractional changes in blood pressure in the experiments where chloralose or chloralose urethan was used as anesthetic The results were essentially as those obtained in barbiturate anesthesia the response to visceral artery occlusions generally being a bradycardia and the carotid artery occlusion response a tachycardia

Occlusion of the abdominal aorta in 4 out of 7 cases produced a slight bradycardia in 2 cases there was no change and in 1 a tachycardia

Relationship between control pressure level and pressor response At the end of the barbiturate experiments the author became aware of the fact that the increments in the systemic arterial pressure during visceral artery occlusions seemed to be dependent on the level of the control pressure In the barbiturate series the control pressures were on an average 146 mm Hg (range 115—173 mm Hg) Considering separately the occlusions performed at control pressures below 146 mm of Hg and those performed at pressures above this value the average ratio between the pressure during the occlusion and the control pressure amounted to 1 18 and 1 03 respectively

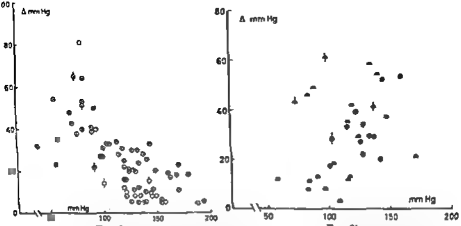

Fig 2a Fig 2b

Fig 2 a Pressure increments (ordinate) against control pressure (abscissa) Combined occlusions of coeliac and superior mesenteric arteries in the 8 chloralose or chloralose urethan experiments Symbols as in Fig 1

Fig 2 b Pressure increments against control pressures of the carotid artery occlusions performed on the same cats as in Fig 2 a Symbols as in Fig 1

For further elucidation of this relationship the author decided to perform visceral artery occlusions at control pressures which were purposely set at different levels by intravenous injections of small amounts of pentobarbital or by withdrawal or infusion of blood These experiments were all performed in cats anesthetized with chloralose as a consequence of these manoeuvres the range of control pressures (53—158 mm Hg) was greater and the average (119 mm Hg) was lower in the chloralose anesthetized animals than in the pentobarbital series

The results of these variations in the control pressure are seen in Fig 2 a where the increments caused by the combined occlusion of the coeliac and superior mesenteric arteries of all the chloralose experiments are plotted against their control pressures The average increment at occlusions performed at control pressures below 100 mm Hg was 45 mm Hg at control pressures above 100 mm Hg the average increment was 17 mm Hg

Fig 2 b shows the corresponding plots for carotid artery occlusions here the relationship is the reverse i e the greatest pressor responses are generally associated with the highest control pressures

Fig 3 and 4 show increments against control pressures in 2 expts performed on respectively a pentobarbital anesthetized and a chloralosed cat The blood pressure was varied by means of controlled bleeding and reinfusion and on each level both visceral and carotid artery occlusions were performed The tendency to get low carotid response and high visceral response with low control pressure and the opposite with high control pressure is obvious However if the control pressures were very low the response to visceral artery occlusion tended to be low From Fig 3 (left) it may be seen that the responses to 2 occlusions performed at control pressures 65 and 84 mm Hg too were rather low these occlusions were made a short time after the animal had been in a state of manifest shock for a few minutes

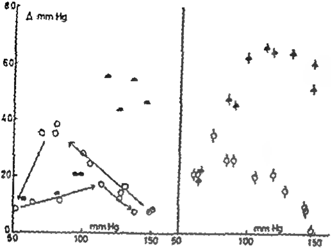

Fig 3 Pressure increments against control pressures of 1 pentobarbital anesthetized cat Left Vagus nerves intact the arrows indicate the sequence in which the occlusions were performed Right Vagus nerves cut Symbols as in Fig 1

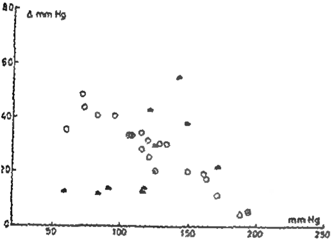

Fig 4 Pressure increments against control pressures of 1 chloralose experiment Symbols as in Fig 1

Discussion

Changes in heart rate In the work of Sarnoff and Yamada (1959) the heart rate was continuously recorded with a Waters cardiotachometer With the vagi intact occlusion of the coeliac superior and inferior mesenteric arteries caused tachycardia in 8 expts bradycardia in 1 and a mixed reaction with late bradycardia in 2 expts After vagotomy was performed tachycardia was observed in 3 of the 4 cats subjected to occlusion of the same arteries in the fourth there was no change in heart rate The tachycardia was most often more pronounced during visceral artery than during carotid artery occlusion whether the vagus nerves were intact or cut

However Selkurt and Rothe (1960) found that slight decreases in heart rate usually accompanied the pressor response provoked by the combined occlusion of the coeliac and superior mesenteric arteries in dogs the rate decreased from an average of 163 to 151 per minute but the difference was not considered statistically significant In the

vagotomized and sinus denervated dogs the average rates before and during occlusion did not differ.

Heymans, De Schaepdryver and De Vleeschhouwer (1960) reported that occlusion and desocclusion of the coeliac and the superior and inferior mesenteric arteries (separately or combined) had no significant effect upon the heart rate in dogs.

Also the results of the present work are inconsistent with those of Sarnoff and Yamada as occlusion of the coeliac and superior mesenteric arteries generally provoked a slight bradycardia. Only at [illegible] occlusions (in different experiments) was there a slight increase in the heart rate. The increase occurred either at the first occlusion of an experiment or in occlusions in which the control period had been very long; the lightly anesthetized animals showed in these instances symptoms of excitation when the ligatures were tightened around the arteries. In the author's opinion irritation of the peritoneum provoked in these occlusions a sympathetic outburst great enough to convert the general tendency to bradycardia into a tachycardia. In support of this interpretation it may be mentioned that in several experiments it was observed that mechanical irritation of the peritoneum produced a rather pronounced tachycardia.

Thus, bradycardia has been the most commonly reported finding, and it is reasonable to interpret it as a reflex caused by the pressure rise and elicited in baroreceptors outside the visceral arteries (mainly the sino-cardio-aortic region). In this way the bradycardia harmonizes with the peripheral resistance fall during visceral artery occlusion demonstrated in a previous work (Nissen 1964).

Relationship between control pressure level and pressor response. Both the pressure increments due to carotid artery occlusions and to mesenteric artery occlusions were found to be highly variable. One of the reasons for this variation is a dependence on another parameter, the control pressure.

In the case of the carotid occlusions this dependence is well known. Thus, Prochnik, Maison and Stutzman (1950) and Hardman *et al.* (1959) reported that in the dog the pressure increments due to carotid artery occlusion were higher the greater the control pressure, in accordance with the threshold concept for the baroreceptors. The relation was nearly linear cutting the control pressure axis at about 60 mm Hg in the work of Prochnik *et al.* In the work of Hardman *et al.* the linearity was limited to control pressures between 100 and 160 mm Hg; below 100 mm Hg the curve was convex towards the control pressure axis.

Bevan, Prior and Verity (1961) arrived in all essentials at the same conclusion from experiments in cats.

The present work is in accordance with these papers: from Fig. 2 b, 3 and 4 it may be seen that a fall in carotid artery response with falling control pressures is the rule. (Yet in 4 occlusions of the carotid arteries in chloralosed cats with systemic pressures below 100 mm Hg a relative high pressor response was obtained; the author is unable to offer an explanation; a similar phenomenon observed by Bevan *et al.* in chloralosed cats was interpreted as carotid chemoreceptor activity.)

In the case of visceral artery occlusions a nearly linear relation between the pressure increments and the control pressures was also observed, but here the highest increments occurred at the lowest control pressures (Fig. 2 a, 3 and 4). Thus there seems to be a fundamental difference between the responses to carotid artery occlusions and to visceral artery occlusions in this respect. At low control pressures (below about 110 mm Hg) the answer to visceral artery occlusion most often was higher than that to carotid artery occlusion; the opposite was the case at high control pressures. Evidently the

relationship between the visceral artery pressor response and the control pressure level does not fit with a threshold concept similar to that for the carotid sinus baroreceptors

In the light of the observed relationships a number of observations reported in the literature may be subject to reinterpretation

Sarnoff and Yamada observed that the pressure increments during visceral artery occlusion often exceeded those observed during carotid artery occlusion and claimed — with som hesitation — that the carotid sinus baroreceptors in the cat were domina ted by the hypothetical visceral baroreceptors From the figures in their paper the following control pressures for all the occlusions of the coeliac superior mesenteric and inferior mesenteric arteries (combined or separate) may be read 150 100 90 80 80 75 and 85 mm Hg The control pressures for the carotid artery occlusions were 65 90 90 and 75 mm Hg Except for the 150 mm Hg pressure these pressures should in the present author s experience lead to high responses to visceral artery occlusions and low responses to carotid artery occlusions

Selkurt and Rothe has also reported that the pressure increments during visceral artery occlusion are great and exceed those produced by carotid occlusion However from their data the average control pressure in the 4 cat expts may be calculated to have been as low as 72 mm Hg

From a series of dog experiments the same authors concluded that in these animals the pressure increment in response to combined occlusion of the coeliac and superior mesenteric arteries is low (average 13 mm Hg) in comparison with the response to bilateral carotid artery occlusion (average 23 mm Hg) However it may be calculated from their data that in this series of experiments the control pressure was rather high (135 mm Hg average for 6 expts)

Neither Sarnoff and Yamada nor Selkurt and Rothe have commented upon the possible effect of the control pressure level on the results but in the opinion of the present author it may have been a factor of considerable importance

Then there is perhaps no basis for the claim of a species difference In this context attention may also be directed to the fact that in dog experiments Heymans *et al* obtained much bigger increments in arterial pressure upon occlusion of the coeliac and mesenteric arteries than those observed by Selkurt and Rothe however no information on the control pressure were given

The nature of the pressor response to occlusion of the visceral arteries Only Yamada (1962) have been able to obtain systemic pressure fall in the dog by perfusion of the mesenteric vascular bed under high pressure (from a donor dog) Yamada states that the pressor response obtained by occlusion of the superior mesenteric and coeliac artery in the dog deprived of the sino-aortic reflexes is a reflex phenomenon with receptor organs possibly in the area of the mesenteric arteries

Gammon and Bronk (1935) were unable to demonstrate an effect on the general blood pressure resulting from perfusion of the superior mesenteric artery at different pressures

Bauereisen Lutz and Peiper (1963) perfused alternately in the cat the superior mesenteric and the femoral artery with a constant output pump in order to exclude hemodynamic artefacts They concluded that it was not possible by variation of the intraluminal pressure in the superior mesenteric artery to obtain reproducible changes in the systemic pressure

Boyer and Scher (1960) conclude from their experiments on cats that no effects in the systemic pressure could be evoked by means of perfusing the abdominal aorta or

the superior mesenteric artery with wide variations in the perfusion pressure

Onnis Shumacker and Bounous (1963) found that the pressor response to occlusion of the coeliac and superior mesenteric artery was not reduced in dogs by thoraco-lumbar sympathectomy and splanchnicectomy Since the hypothetical baroreceptor zones should be denervated by these operations they suggested that the hypertensive reaction is not due to reflex vasoconstriction but to a change in the distribution of blood flow and a reduction in the effective vascular bed

In view of the negative results obtained by several authors with several different methods it is reasonable to conclude that the pressor effect caused by visceral artery occlusion is not a reflex phenomenon similar to that produced by carotid artery occlusion

Selkurt and Rothe point out that a part of the rise in pressure following splanchnic arterial blockade could be the hydraulic effect of imposing a fixed resistance to arterial outflow with constant cardiac output In the light of the relative high pressure increments obtained at low control pressures in the present work a mechanism like this would be particularly likely if the blood flow through the coeliac superior and inferior mesenteric arteries amounted to a greater part of the cardiac output, the lower the blood pressure was Sapirstein Sapirstein and Bredemeyer (1960) investigated the distribution of the cardiac output with the indicator fractionating technique in the normal rat and in the rat exposed to a moderate (10 ml per kg) and a severe (21—25 ml per kg) bleeding Their observations showed that the splanchnic blood flow fraction (kidneys not included) is increased some 25 per cent in moderate hemorrhage (blood pressure 90 ± 30 (SD) mm Hg) Severe bleeding reduced the flow fraction a little Several authors have not observed a favouring of the splanchnic blood flow in hemorrhage However even the findings of Sapirstein *et al* are unable to account for more than a part of the great variation in blood pressure increment observed as a function of the control pressure

At the low pressures where the increments are disproportionately high it cannot be excluded that the heart muscle works in a state of light hypoxia If the blood pressure is raised by imposing a resistance to the circulation (for instance by occlusion of the mesenteric arteries) this may start a beneficial circle enhancing the coronary blood flow and with that the power of the heart resulting in an increment in arterial pressure out of proportion to that of the imposed increment in peripheral resistance Likewise a dislocation of portal venous blood into the great veins during mesenteric artery occlusion would represent a blood pressure enhancing factor especially in a state of pre shock where the blood pressure is highly sensitive to small alterations in the effective blood volume

At high control pressures on the contrary a tendency for the cardiac output to fall with increments in arterial pressures (and left ventricular systolic pressure) could possible explain a part of the tendency to get low pressure increments at this level

In view of the essential role played by the baroreceptors buffering the blood pressure the sensitivity of this servomechanism may be of considerable importance as a factor determining the magnitude of the increase in pressure following occlusion of the coeliac and superior mesenteric arteries From present evidence (Koch 1931 cited by Heymans and Neil 1958) it appears that the sensitivity of the carotid sinus is considerably lower at subnormal pressures than at normal pressures Although the effect of the pulse pressure is not taken into consideration in these studies it seems reasonable to assume that the great increments in pressure observed during mesenteric artery occlusions may

in part be explained by a less efficient counteraction of the pressure rise by the classical baroreceptors

The possibility that a sort of venous reflex forcing the blood towards the heart is released by mesenteric artery occlusion was proposed by Selkurt and Rothe This possibility does not seem to have been further examined yet

To exclude that the pressor responses observed in chloralose anesthesia were caused by a reduced sensitivity of the buffering system owing to the depressing effect of chloralose on the sinus baroreceptors (Neil Redwood and Schweitzer 1949) a part of the present experiments were performed in pentobarbital anesthesia At the same control pressures no significant difference in the pressor response could be observed

It must be concluded with certainty that the pressor responses seen following occlusions of the coeliac and superior mesenteric arteries is not a reflex phenomenon similar to the carotid sinus reflex It is not caused by vasoconstriction in the resistance vessels and a stimulation of the heart as is the carotid sinus response indeed it is produced in spite of a peripheral vasodilatation and a slight bradycardia

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Acta physiol scand 1965 63 77—83

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Effect of Nicotine and Nicotine Analogues on Tissue and Urinary Catecholamines in the Rat

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Abstract

WESTFALL T C *Effect of nicotine and nicotine analogues on tissue and urinary catecholamines in the rat* Acta physiol scand 1965 63 77—83 — The time response pattern of nicotine and the nicotine analogue 3 pyrrolidino-methylpyridine (W 16) on the noradrenaline (NA) content of heart spleen kidney and liver was studied following intraperitoneal administration in rats It was found that both compounds produced a transient but significant decrease Nicotine produced an effect that was visible within 5 min with the NA content returning to control levels in 60—180 min Although quantitatively different, W 16 produced an effect qualitatively similar to nicotine The effect of nicotine and several nicotine analogues on the 24 hour urinary excretion of catecholamines was also studied in rats Three out of four analogues investigated produced a slight increase in the excretion of adrenaline (A) and NA at high dose levels (5—10 mg/kg) At a dose of 1 mg/kg nicotine produced a significant increase in the 24 hour urinary excretion of A but not NA

Although the effects of nicotine on sympathetic ganglia date back to the time of Langley (1889) and that it is rather well known that nicotine causes a release of adrenaline (A) and noradrenaline (NA) from the adrenal medulla (Cannon and Hoskins 1912 Stewart and Rogoff 1919 Segawa 1934 Woods and Richardson 1955 and Kiser Booher and Watts 1955) there have been few reports on the effect of nicotine on tissue catecholamine levels In the present study the effect of nicotine and a nicotine analogue 3-pyrrolidino-methyl pyridine (W 16) on the NA content of heart spleen kidney and liver was investigated at various times following i p administration in rats The effect of some synthetic nicotine analogues on the 24 hour urinary excretion of A and NA in the rat were also studied

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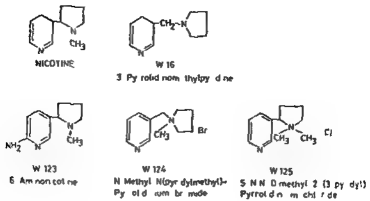


Fig 1 Chemical formulae of nicotine and four nicotine analogues

TABLE I Nicotine analogues on urinary catecholamine excretion in rats (cf Fig 1) (Urine collected from 4 rats)

	Control (n=10)		0 05—0 1 mg/kg (n=4)		0 —1 0 mg/kg (n—4)		5 0—10 0 mg/kg (n—4)	
Compound	NA¹	A¹	NA	A	NA	A	NA	A
W— 6	3 03	0 74	4 01	0 58	4 48	0 63	5 01	1 0
W—123	1 80	0 40	2 11	0 45	2 10	0 43	2 79	0 35
W—124	1 83	0 65	2 79	0 81	3 58	0 63	4 13	2 09
W—125	2 30	0 63	2 63	0 72	2 31	0 63	3 04	1 14

¹ NA and A values expressed as μg/kg/24 hrs

Methods

Rats of the Sprague Dawley strain weighing from 250—350 g were used For the urine determinations rats were placed in individual metabolism cages and allowed 4—5 days for acclimatization Food and water were allowed *ad libitum* during the experiments and special care was taken to keep food and feces separated from the urine during coll ction Normal hydrochloric acid was added to the urine as preservative The effect of 6 different doses ranging from 0 05 to 10 0 mg/kg of 4 nicotine alkaloids injected i p was studied on the 24 hour urinary A and NA excret on Follo ing these initial experiments one of the alkaloids W 16 (Fig 1) together with nicotine was chosen for d tailed studies The effect of one selected dos of each of these drugs on the 24 hour excretion of A and NA was studied in ord r to prov de enough data for a statistical comparison

A chronic experiment was carried o it in which nicotine (1 mg/kg i p) was injected once per day and urine collected and analyzed on days 2 7 8 and 13

For th tissue studies nicotine (0 5 and 1 0 mg/kg) and W 16 (5 mg/kg) were injected i p and the rats sacrificed by a blow on the head at 5 15 30 60 180 and in some cases 1440 min A and NA determinations were carried out on heart spleen

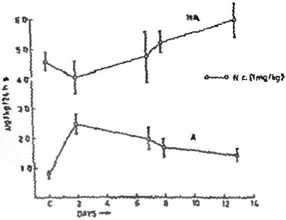


Fig 2 Chronic administration of nicotine (1 mg/kg i p) injected once per day on the 24 hr urinary excretion of A and NA in the rat Each point represents the mean excretion from 10 rats

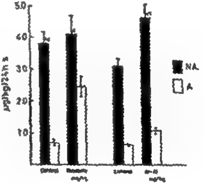


Fig 3 Effect of nicotine (1 mg/kg) and W 16 (5 mg/kg) injected i p on the 24 hr urinary excretion of A and NA in the rat. Number above columns represent number of rats

kidney and liver In the case of heart and liver 2 of each were pooled whereas 4 spl ens and 4 kidneys were generally pooled for determination The tissues were extracted with trichloracetic acid filtered adsorbed on alumina and catecholamine determinations carried out according to the method of Euler and Lishajko (1961 All samples were read in a Coleman fluorometer and the results given in terms of the bases Urine samples were filtered adsorbed on alumina and determined in the same manne as above No correction was made for the 10 to 20 amines lost during the procedure

Results

Table 1 shows the results of 4 nicotine alkaloids the chemical formulae of which are represented in Fig 1 on the 24 hour urinary excretion of A and NA in the rat The range of doses studied falls into 3 categories 0 05—0 1 0 5—1 0 and —10 mg kg Each category represents the excretion from 4 rats It appears that with a dose of 5—10 mg kg there was a slight inc ease in the urinary excretion of A and possibly that of NA with W 16 124 and 125 The most active compound seemed to be W 16 even at the smaller dose range It exerted a greater effect on NA than the A excretion

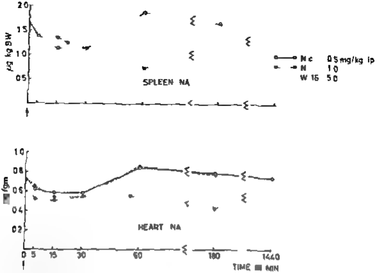


Fig 4 Effect of nicotine (0 5 and 1 0 mg/kg) and W 16 (5 mg/kg) injected i p on NA content of heart and spleen Abscissa represents time in min following injection Each circle represents mean of 4 determinations

During all these experiments each group of rats served as its own control prior to administration of the drugs

The effect of the chronic administration of nicotine in a dose of 1 mg/kg/day i p is depicted by Fig 2 As can be seen nicotine produced a significant increase in the A excretion levels The greatest response was seen on day 2 ($P < 0\ 001$) although there was still a significant increase on days 7 8 and 13 ($P < 0\ 01$) Nicotine had no significant effect on the excretion of NA

Fig 3 shows the effect of nicotine (1 mg/kg) and of W 16 (5 mg/kg) injected i p on the 24 hour urinary excretion of A and NA Nicotine produced a significant increase in the A level ($P < 0\ 001$) while it had no significant effect on the 24 hour excretion of NA W 16 on the other hand produced a significant increase in both the 24 hour excretion of A ($P < 0\ 05$) and NA ($P < 0\ 01$)

The time response pattern of two different doses of nicotine and one of W 16 on the NA content of the heart and spleen is shown in Fig 4 With both doses of nicotine there was a moderate decrease in the NA content of the heart visible within 5 min and reaching a maximum in 15 min The maximum effect was a decrease to 80 and 69 % of control levels for the 0 5 and 1 0 mg/kg dose respectively The level remained low for 30 min returning to a level slightly higher than in the control at 60 min and to pre injection levels by 180 min With W 16 there was a slight decrease of the amine content of the heart seen within 15 min and lasting for 180 min The NA levels in the heart were back to pre injection levels by 24 hours

The effect of these 2 compounds on spleen NA was somewhat greater than the effect on the heart although the same pattern was seen Nicotine again produced an effect visible within 5 min with a decrease to 68 % of control in 30 min Similar to the effect on the heart there was a greater than normal tissue value seen at 60 min with a return

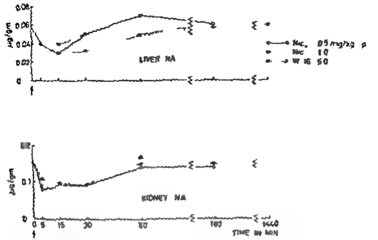


Fig. 5 Effect of nicotine (0.5 and 1.0 mg/kg) and W 16 (mg/kg) injected i.p. on NA content of liver and kidney. Abscissa represents time in min following injection. Each circle represents mean of 4 determinations.

to pre-injection values in 180 min. W 16 was much slower in producing its maximum effect (45 % of control) which was seen in 60 min.

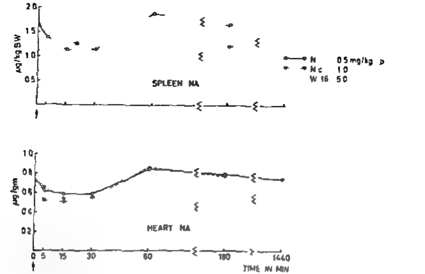
Fig. 5 shows the effect of both doses of nicotine and of W 16 on the NA content of kidney and liver. With regard to the kidney, nicotine in the dose of 0.5 mg/kg and W 16 produced a decrease in the amine levels to a value of 53 % of control levels. The response was visible in 5 min for nicotine and 15 min for the other compound. Nicotine in the dose of 1.0 mg/kg produced a decrease to 63 % of control with the maximum effect appearing in 15 min.

Both compounds produced a similar response on liver NA levels visible within 5 min. The maximum effect was a decrease to 60 % of control levels.

An analysis of the A content of these various tissues was also carried out but because of the small amounts of this amine normally present in the tissues the data are not presented.

Discussion

Quantitative information on the activity of the sympathetic adrenergic system and the adrenal medulla can be obtained by estimating the urinary output of A and NA. This type of procedure was used to provide data on the action of nicotine as well as of nicotine analogues. It was found that 3 out of 4 nicotine analogues appeared to produce a slight increase in the urinary excretion of A and possibly NA in the rat, indicating that they have an action at least in part similar to nicotine itself. When studied chronically, it was found that nicotine produced a significant increase in the A but not NA lasting for the whole period of injections (13 days). This effect of nicotine on catecholamine excretion substantiates that is found in rats after subcutaneous



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This work was supported in part by a National Heart Institute postdoctoral fellowship Fa-HL-A, 331—01 Grant AF EOAR 64—31 from the Air Force Office of Scientific Research. OAR, through the European Office Aerospace Research, United States Air Force, and the Swedish Tobacco Co Stockholm. The author wishes to express his appreciation to Dr F Haglid for placing the nicotine analogues at my disposal.

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Energy Cost of Alternating Positive and Negative Work

By

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Abstract

Hesser C. M *Energy cost of alternating positive and negative work* Acta physiol scand 1965 63 84—93 — The energy cost of walking up and down a Λ-shaped 1 m high staircase was studied in 10 male and 10 female subjects in terms of the O_2 requirement for performing the work under truly aerobic conditions Two different speeds of walking were used yielding external work loads of $k \times 14.7$ and $k \times 26.7$ kpm/min (k = body weight of individual in kg) in both the positive and negative phases At the lower speed the net O_2 cost per kg b w averaged 16.8 ml min independent of sex at the higher speed the corresponding value was 33.0 ml/min in the men and 32.0 ml/min in the women From these values the average physiological load was estimated to $k \times 8.2$ kpm min for men and women at the lower speed and to $k \times 16.2$ and $k \times 15.7$ kpm min for men and women, respectively at the higher speed The ratio of O_2 costs for the positive and negative works approximated 8 : 1 at the lower speed and 5 : 1 at the higher speed During each period of positive work the calculated O_2 demand of a 70 kg man exceeded the O_2 supply to the working muscles by about 60 ml at both speeds Thus O_2 debt of 60 ml could be contracted without any increment in the lactacid O_2 debt and supports the notion that a certain amount of O_2 can be released from the myoglobin O_2 store before anaerobic processes become involved The O_2 capacity of this aerobic debt mechanism was calculated to be in the order of 3 ml per kg of active muscle tissue Evidence is presented that, in the steady state the upper limit for performing work under truly aerobic conditions corresponds to about 70 per cent of the maximal O_2 consumption

Walking up and down a staircase a number of times implies the performance of alternating positive and negative work. If the speed of walking is kept constant the external work load is numerically the same in the up and down phases although with different signs, and can be calculated by applying simple physical laws The energy expenditure as measured by the O_2 consumption is much less in negative than in positive work Abbott, Bigland and Ritchie 1952 Abbott and Bigland 1953 Asmussen 1953) The present study was designed to determine the energy cost of rapidly alternating positive and negative work under truly aerobic conditions as compared with the energy cost of continuous positive work.

Methods

Subjects The experiments were performed on 10 male and 10 female subjects who were physically active but did not take part in athletics Age body weight and height are given in Table I Prior to the investigation all subjects underwent a clinical examination including clinical history auscultation of heart and lungs X ray of the chest ECG and routine blood and urine analysis They showed no signs of pulmonary or cardiac disease

Procedure To achieve a rapidly alternating positive and negative work the subject walked up and down a staircase (see below) at a constant speed the rate being set by a metronome Two series of experiments were made the first series at a speed of 160 steps/min the second series at 88 steps/min The duration of each single period of positive or negative work was 2.25 sec at 160 steps/min and 4 1 sec at 88 steps/min The work produced or absorbed in the 2 types of work was calculated as the product of body w in kg (k) times the vertical distance it was lifted or lowered. At 160 steps/min this distance was 26 7 m/min. The external load thus amounted to $k \times 26.7$ kpm/min in each one of the 2 phases of work At 88 steps/min the corresponding loads were $k \times 14.7$ kpm/min

All experiments were done in the morning After a light breakfast the subject rested in the reclining position for at least 45 min The resting metabolic rate was then determined over a 5 to 10 min period by means of a Krogh spirometer placed close to the staircase In the experiments at 160 steps/min all subjects then made 2 runs in the staircase separated by a rest period of at least 40 min The number of completed rounds in the staircase was 26.5 in the first run, and 40 in the second run the work periods thus lasting for 3 min 59 sec and 6 min respectively (hereafter called the 4 min and 6-min runs) During the last minute of each run expired air was collected in a Tissot spirometer (see below) Immediately after the work had stopped the pulse rate was determined and the subject was allowed to rest in the reclining position The O uptake was then continuously recorded from the beginning of the 2nd to the end of the 10th min of recovery by means of the Krogh spirometer 30 min after the 4 min run the metabolic rate was again determined to check that the rest period was sufficiently long for restitution of normal resting values This was always the case On a second day usually a few days later the subject made the same 2 runs but this time expired air was collected during the 1st min of recovery of each run. In addition the O uptake was measured continuously from the beginning of the 3rd to the end of the 10th min of recovery using the Krogh spirometer

The experiments at 88 steps/min were performed on 5 men and 5 women all of which had also participated in the experiments at 160 steps/min The same general procedure was used except that the numbers of completed rounds in the staircase now were 14 5 and 22 and the duration of the work periods 3 min 57 sec and 6 min respectively in the 2 runs (hereafter called the 4 min and 6-min runs)

Design of the circular staircase The staircase was of the type used in the Nylin heart function test (Nylin 1933 1938) Instead of walking up and down the same staircase the subject walks along a circular pathway of 1 1 m radius The pathway includes two Λ shaped staircases so that for completion of a single loop the subject has to walk upstairs and downstairs 2 times Each staircase has a semi-circular shape if seen from above The profiles of the staircases if unfolded, are symmetric and identical each with 6 steps upwards and 6 steps downwards and with the summit step 1 m above the lowest step at floor level

Respiratory minute volume and O uptake during the work periods were measured by collecting expired air in a 100 l Tissot spirometer placed in the center of the staircase on a disk which could be passed ... and connected by low resistance smooth bore tubing to a low resistance valve assembly with a dead space of about ... ml The ... was thoroughly mixed before samples were taken and analyzed in duplicate for O and CO with a modified Haldane apparatus.

The lactacid and alactacid components of the O debt (Margaria Edwards and Dill 1933) were determined by analyzing in detail the O uptake data obtained in recovery employing the method described by Henry and DeMoor (1950) The individual O recovery curves were plotted semilogarithmically using the rate of O uptake (in excess of resting rate as a function of time The magnitudes of the 2 exponential components of the O debt were then computed from the curve constants obtained from the semilogarithmic plots

The aerobic work capacity i the maximal O uptake per minute was calculated from the O uptake and pulse rate at submaximal work by using the nomogram of Åstrand (1960) The values for O uptake and pulse rate were determined as the means of the values obtained in the last minute of the 4 min and 6-min runs For reasons of convenience the pulse rate was deter

Table I Data from experiments at 160 steps/min

Variable		Men	Women	Difference Men Women
Number of subjects		10	10	
Age years		21 5 (19—26)	23 9 (20—37)	
Height cm		177 7 (172—186)	166 9 (160—178)	
Weight (including light clothes) kg		68 3 (61 0—76 0)	59 7 (53 3—69 7)	
Net O_2 uptake during last minute of 4 min runs	ml/min STPD	2 197±54 170	1 825±46 146	+372 ±71 P < 0 001
	ml/kg min STPD	32 2±0 41 1 30 CV =4 0	30 6±0 37 1 16 CV =3 8	+1 6 ±0 55 P < 0 01
Net O_2 uptake during last minute of 6 min runs	ml/min STPD	2 227±57 179	1 859±49 156	+368 ±75 P < 0 001
	ml/kg min STPD	32 6±0 52 1 64 CV=5 0	31 1±0 46 1 44 CV =4 6	+1 5 ±0 69 P < 0 05
Total O_2 dept contracted in 4-min runs	ml/kg STPD	31 7±1 2 3 65	36 9±1 7 5 45	—5 2 ±2 1 P 0 05
Total O debt contracted ^ min runs	ml/kg STPD	34 1±1 7 5 39	40 7±2 3 7 28	—6 6 ±2 9 P 0 05
Pulse rate immediately after work	beats per min	161 4±2 4 7 6	172 8 ±3 0 9 3	—11 4 ±3 8 P < 0 01
Maximal O uptake[1]	liter/min STPD	3 25±0 10 0 33	2 63±0 09 0 29	+0 62±0 14 P < 0 001
	ml/kg min STPD	47 6±1 2 3 65	44 2±1 4 4 42	+3 4 ±1 8 P 0 05
Gross O uptake during exercise in per cent of maximal O uptake		75 9±1 9 6 1	77 8±2 3 7 3	— 1 9 ±3 0 P 0 1

Figures denote means ± standard errors of the means and standard deviations Figures in brackets denote ranges CV = coefficient of variability P= level of significance

[1] Calculated from pulse rate and O uptake during exercise by the aid of nomogram of Åstrand (1960)

mined immediately after work by measuring the exact time for 15 pulse beats This seems justifiable since the pulse rate does not change appreciably during the first 5—10 sec after work (Christensen Hedman and Saltin 1960 Barr *et al* 1964)

Lactic acid determinations were made by using a modification of the colorimetric method of Barker and Summerson (1941) as described by Strom (1949) The blood samples were taken from the warmed up finger tip just prior to and 1/2—2 min after the runs

Barometric pressure averaged 760 0 mm Hg (range 743—776) and room temperature 23 0 C (range 20—26)

Results

Experiments at 160 steps/min

Mean values and standard errors of the means for the data obtained in 10 men and 10 women are given in Table I The values for net O_2 uptake (i e in excess of resting O_2 consumption) in the last minute of the 4 min and 6-min runs were significantly higher in the male group not only when expressed in absolute values but also when computed in relation to body weight In both groups the net O_2 uptake in the 6th min (mean 32 6 ml/kg in the males 31 1 ml/kg in the females) was somewhat larger than in the 4th min (mean 32 2 and 30 6 ml/kg respectively) The difference was statistically significant ($P < 0\ 05$) in the female group when calculated on intraindividual differences but not in the male group indicating that in the men the O uptake had attained an approximately stable level by the 4th min of exercise

The total O_2 debt showed a small but significant increment ($P < 0\ 05$) in both groups when the work period was increased from 4 min to 6 min (mean increase 2 4 and 3 8 ml/kg in the males and females respectively) indicating that in neither group the O_2 uptake covered the O_2 demand in the 5th and 6th min of work The O_2 debt was significantly larger in the females both in the 4 min and 6-min runs (Table I)

The calculated aerobic work capacity was higher in the men also when computed in relation to body weight (mean 47 [illegible] and 44.2 ml O_2/kg min in the males and females respectively) The relative working intensity during the runs was somewhat less in the men corresponding to 76 per cent (range 67—87) of the aerobic work capacity as compared to 78 per cent (range 63—89) in the women

The blood lactate concentration was determined in two of the male and two of the female subjects For the males the maximal increase in lactic acid concentration averaged 9 mg per 100 ml blood in the 4 min runs and 16 mg per 100 ml blood in the 6-min runs The corresponding figures for the females were 31 and 36 mg per 100 ml blood The means of the working intensities of the two men and two women corresponded to 73 and 78 per cent of the aerobic capacity

Experiments at 88 steps/min

Table II summarizes the means of the data from the experiments at 88 steps/min which were performed on 5 males and 5 females In neither group did the net O_2 uptake in the last minute of the 6-min runs differ significantly from that in the last minute of the 4-min runs indicating that in both groups the O_2 uptake had attained a stable level by the 4th min of exercise The mean of the two values was therefore taken as a measure of the excess O_2 uptake in the stable state of exercise When calculated in relation to b w there was no significant difference in net O_2 uptake between the 2 groups the mean value for all 10 subjects being 16 8 ml/kg min (range 15 6—18 7)

TABLE II Data from experiments at 88 steps/min

Variable		Men	Women	Difference Men Women
Number of subjects		5	5	
Weight (including light clothes) kg		70.1 (66.8–76.0)	59.3 ([illegible]4.7–67.0)	
Net O_2 uptake average for last minute of the 4 min and 6 min runs	ml/kg min STPD	17.5±0.56 1.25 CV = 7.1	16.2±0.22 0.50 CV = 3.1	+1.3±0.60 P > 0.05
Total O_2 debt contracted in 4 min runs	ml/kg STPD	15.0±0.8 1.85	14.9±0.4 0.84	+0.1±0.9 P > 0.1
Total O_2 debt contracted in 6-min runs	ml/kg STPD	14.8±1.0 2.31	15.3±0.7 1.51	−0.5±1.2 P > 0.1
Gross O_2 uptake during exercise in per cent of maximal O_2 uptake		47.7±2.7 6.0	43.1±2.6 5.7	+4.6±3.8 P 0.1

Figures denote means ± standard errors of the means and standard deviations Figures in brackets denote ranges CV = coefficient of variability P level of significance

The total O_2 debts contracted in the 4 min and 6 min runs did not differ significantly either within each group or between the two groups of subjects (Table II)

The relative working intensity in the males and females respectively corresponded to 48 per cent (range 39—54) and 43 per cent (range 34—48) of the aerobic work capacity The maximal increment in blood lactic acid concentration was determined in 4 females and averaged 5 mg per 100 ml in the 4 min runs and 6 mg per 100 ml in the 6 min runs

Discussion

Oxygen cost and mechanical efficiency

From the observation that at the lower load (88 steps/min) the O_2 debt and blood lactate showed no increments when the work period was increased from 4 min to 6 min it may be inferred that after the 4th min the energy expenditure was entirely covered by the O_2 supply It follows then that the O_2 requirement of this work under truly aerobic conditions corresponds to the observed stable rate of net O_2 uptake (mean 16.8 ml/kg min)

On extending the work period from 4 min to 6 min at the higher load (160 steps min) the O_2 debt increased significantly indicating that part of the energy expended was delivered by anaerobic processes This was the case also in the male group in which the O_2 uptake levelled off at a stable rate by the 4th min of work The O_2 requirement for performing the work under truly aerobic conditions may be estimated by adding the O_2 consumption equivalent to the anaerobically released energy to the observed

O_2 uptake Since anaerobic work with a subsequent aerobic recovery is only about 50 per cent as efficient as aerobic work (Asmussen 1946) the amount of O equivalent to the anaerobically released energy is only about half as large as the lactacid component of the O_2 debt The observed increment in total O_2 debt after the 4th min was almost entirely due to the lactacid component and amounted to 1.2 and 1 9 ml/kg min in the males and females respectively In the men the measured stable rate of excess O uptake was 32 4 ml/kg min Consequently the O_2 requirement for performing the work under truly aerobic conditions can be estimated to (32 4 + 1/2 × 1.2 =) 33 0 ml/kg min in the average male subject In the women the measured net O_2 uptake in the 6th min was 31 1 ml/kg From the observation that the O_2 uptake during the adaptation to exercise changes as an exponential function of time (Henry 1951) the half time of which is in the order of 30 sec in women (Hesser unpublished observations) the net O uptake in the 5th min of the work was estimated to 31 0 ml/kg Hence for the average female subject the O_2 cost of this alternating positive and negative work under truly aerobic conditions can be calculated to 32 0 ml/kg min The O_2 cost for a given quantity of work was thus somewhat less in the females indicating that women accomplish this type of work with a somewhat higher mechanical efficiency

Since the external load was proportional to the body weight the interindividual variation in O_2 uptake per kg b w should (1) be smaller than the variation in total O uptake and (2) correspond to the variation in mechanical efficiency The first statement is confirmed by experimental results Thus, when calculated on all 20 individual data obtained in the 6th min of work at 160 steps/min the coefficient of variability in net O_2 uptake/kg was 5 6 per cent and in total O uptake 12 2 per cent The mechanical efficiency of walking up and down a staircase cannot be determined because the average load imposed on the subject is unknown It seems reasonable to assume however that the interindividual variation in efficiency should be similar to that found in other types of work in which large groups of muscles are used From the data obtained by e g Wahlund (1948) and Åstrand (1952) it can be calculated that the coefficient of variability in net efficiency of riding a bicycle ergometer at loads of 600–1200 kpm min amounts to about 5 per cent (range 4 3—7 1) Similar values for the coefficient of variability in net O_2 uptake per kg b w were found in the present investigation (Table I and II) The experimental results are thus in accord with the assumption that the interindividual variation in mechanical efficiency of walking up and down a staircase is about the same as that of riding a bicycle ergometer

Physiological work load

Although the external load in each one of the two phases of positive and negative work can be estimated by applying simple physical laws such is not the case with respect to the average physiological load imposed on the subject This follows from the fact that the energy expenditure is less in negative than in positive work as shown by Abbott Bigland and Ritchie (1952) Abbott and Bigland (1953) and Asmussen 1953 These authors found in experiments on the bicycle ergometer that the ratio of O_2 costs for similar rates of positive and negative works increased with speed of pedalling and also that the O_2 cost of negative work remained relatively constant when the rate of work was changed by varying the speed at constant force The experiments were arranged in such a way that the muscle movements were truly comparable in the two directions i e mirror images Since the procedure of walking up and down a staircase is not characterized by such a symmetry of movements the values for the ratio of O_2 costs

TABLE II Data from experiments at 88 steps/min

Variable		Men	Women	Difference Men Women
Number of subjects		5	5	
Weight (including light clothes) kg		70 1 (66 8—76 0)	59 3 (4 7—67 0)	
Net O uptake average for last minute of the 4 min and 6-min runs	ml/kg min STPD	17 5±0 56 1 25 CV =7 1	16 2±0 22 0 0 CV =3 1	+1 3±0 60 P > 0 05
Total O debt contracted in 4 min runs	ml/kg STPD	15 0±0 8 1 85	14 9±0 4 0 84	+0 1±0 9 P > 0 1
Total O_2 debt contracted in 6-min runs	ml/kg STPD	14 8±1 0 2 31	15 3±0 7 1 51	—0 5±1 2 P 0 1
Gross O uptake during exercise in per cent of maximal O uptake		47 7±2 7 6 0	43 1±2 6 5 7	+4 6±3 8 1 0 1

Figures denote means ± standard errors of the means and standard deviations Figures in brackets denote ranges CV =coefficient of variability P=level of significance

The total O_2 debts contracted in the 4 min and 6-min runs did not differ significantly either within each group or between the two groups of subjects (Table II)

The relative working intensity in the males and females respectively corresponded to 48 per cent (range 39—54) and 43 per cent (range 34—48) of the aerobic work The maximal increment in blood lactic acid concentration was determined in 4 females and averaged 5 mg per 100 ml in the 4 min runs and 6 mg per 100 ml in the 6-min runs

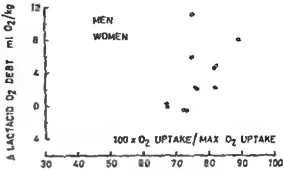
Discussion

Oxygen cost and mechanical efficiency

From the observation that at the lower load (88 steps min) the O_2 debt and blood lactate showed no increments when the work period was increased from 4 min to 6 min it may be inferred that after the 4th min the energy expenditure was entirely covered by the O supply It follows then that the O_2 requirement of this work under truly aerobic conditions corresponds to the observed stable rate of net O_2 uptake mean 16 8 ml/kg min)

On extending the work period from 4 min to 6 min at the higher load (160 steps min) the O_2 debt increased significantly indicating that part of the energy expended was delivered by anaerobic processes This was the case also in the male group in which the O_2 uptake levelled off at a stable rate by the 4th min of work The O requirement for performing the work under truly aerobic conditions may be estimated by adding the O_2 consumption equivalent to the anaerobically released energy to the observed

Fig 1 Change in lactacid O_2 debt (ml O_2 per kg of body weight) during the 5th—6th min of work in relation to relative working intensity (*abscissa*) in 20 subjects (30 determinations) No increment in O_2 debt is seen until the working intensity exceeds about 70 per cent of the aerobic work capacity



The present observations (Fig 1) indicate that in work involving large groups of muscles the upper limit for truly aerobic work under stable state conditions corresponds to about 70 per cent of the maximal O_2 uptake In Fig 1 the individual changes in lactacid O_2 debt caused by increasing the work period from 4 min to 6 min have been plotted against the relative working intensities determined as the ratio of gross O_2 uptake to maximal O_2 uptake No increment in O_2 debt is seen until the O_2 uptake rate exceeds about 70 per cent of the maximal rate and the O_2 debt then shows a tendency to increase with the relative intensity of the work From the data obtained by Christensen and Hogberg (1950) it appears that also in well trained individuals the upper limit for truly aerobic work corresponds to about 70 per cent of the maximal O_2 uptake

Oxygen requirement versus oxygen supply to the working muscles

Because of the short duration of each single period of positive or negative work (4 1 sec at 88 steps/min 2 25 sec at 160 steps/min) any cyclic variation in cardiac output and in rate of O_2 uptake from the lungs should be rather small (*cf* Barr *et al* 1964 Henry and DeMoor 1956) It may be concluded then that in each single work period the quantity of excess O_2 taken up by the working muscles approximated that taken up from the lungs and also that this quantity was about the same in the two phases of work From the observed rates of O_2 uptake this O_2 quantity for a man weighing 70 kg can be calculated to 85 ml at 160 steps/min and 83 ml at 88 steps/min For the same subject the estimated extra energy expended in each period of positive work (lifting of the body 1 m) corresponds to an oxygen demand of 143 ml at both speeds assuming a mechanical efficiency of 23 4 per cent and a caloric coefficient for oxygen of 4 8 Thus during each period of positive work the O_2 demand exceeded the O_2 supply to the working muscles by about 60 ml at both speeds

All or the main part of this O_2 debt of 60 ml must have been repaid during the ensuing period of negative work otherwise the O_2 debts contracted in the single periods of positive work would have accumulated and given rise to a substantial increment of the total O_2 debt when the work was prolonged by 2 min No such increment was observed at 88 steps/min whereas at 160 steps/min only the lactacid component of the O_2 debt showed a very slight increment It may then also be concluded that the energy corresponding to the O_2 debt of 60 ml was almost entirely delivered either by truly aerobic processes or else by anaerobic processes involved in the contraction of the alactacid component of the O_2 debt The last mentioned possibility may be ruled out

however if as shown by Asmussen (1946) the efficiency of anaerobic work with a subsequent aerobic recovery is only about half as great as the efficiency of aerobic work If so about 120 ml of oxygen would be required to pay back an oxygen debt of 60 ml contracted under anaerobic conditions This quantity is far greater than the calculated amount of 85 ml O_2 that was taken up by the working muscles in each period of negative work

If this reasoning is correct it follows that there must exist a mechanism by which an O_2 debt of at least 60 ml can be rapidly contracted and repaid without causing anaerobiosis in the working muscles It seems reasonable to assume that the myoglobin plays an important rôle in this connection As shown by Theorell (1934) the oxygen dissociation curve of myoglobin is hyperbolic in shape and so related to the O_2 tension that on lowering the O_2 tension from e g 20 to 5 mm Hg the myoglobin O_2 saturation falls from about 90 to 60 per cent From the observations of Chance (1957) it appears that the critical O_2 tension above which the cytochrome system of active tissues can operate at an undiminished rate is in the order of 5 mm Hg It may then be inferred that above this level the energy expenditure is covered by aerobic processes From the data presented by Biörck (1949) the myoglobin bound O_2 in the working muscles can be estimated to about 200 ml at full saturation assuming 20 kg of active muscles A fall of the O_2 tension in the working muscles from 20 to 5 mm Hg would thus result in the liberation of about 60 ml of O_2 from the oxymyoglobin or 3 ml per kg of active muscles In addition about 10 ml of O_2 would be mobilized from the oxygen stored in physical solution

Theoretical considerations thus suggest that in alternating positive and negative work an O_2 debt of about 70 ml can be rapidly contracted and repaid without causing anaerobiosis provided the average working intensity is not high enough to cause the maximum O_2 tension in the muscles to fall below 20 mm Hg At higher working intensities the O_2 capacity of this aerobic debt mechanism will probably become reduced because of a lower maximum O_2 tension in the active muscles This would explain the present observation that on prolonging the work period from 4 min to 6 min, the lactacid component of the O_2 debt did not increase at the lower work rate but showed a small increment at the higher work rate

In experiments on intermittent positive work Åstrand *et al* (1960) obtained evidence suggesting that in man about 430 ml of oxygen may be supplied to the working muscles from oxymyoglobin before anaerobic processes take place In view of the theoretical considerations outlined above this value of 430 ml O_2 seems rather high even considering that the subject studied was in a physically well trained condition and thus as pointed out by the authors probably had a higher than average myoglobin concentration

The experimental part of this study was carried out at the Heart Cl[illegible] f Sodersjukhuset Stockholm I am greatly indebted to the late Professor Gustav Nylin who kindly put the facilities of his laboratory at my disposal My thanks are also due to Mrs M [illegible] and Miss U Granlund for most valuable and skilful technical assistance

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The Effect of Some Physiological Vasodilators on the Vascular Bed of Skeletal Muscle

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Abstract

Kjellmer I and H Odelram *The effect of some physiological vasodilators on the vascular bed of skeletal muscle* Acta physiol scand 1965 63 94—102 — The effects of i a infused ACh ATP histamine and bradykinin (and kallidin) were studied on the isolated calf muscle preparation of cats and compared with the vascular response to graded exercise elicited by stimulating the sciatic nerve With a plethysmographic technique the effects on the resistance vessels the capillary filtration coefficient (CFC) and the capacitance vessels were assessed and through a more indirect approach the occurrence of an increased capillary permeability was estimated All procedures decreased flow resistance ACh and ATP increased CFC approximately as much as exercise and like exercise without any signs of increased capillary permeability As a contrast bradykinin and particularly histamine produced higher CFC values at every of diminished flow resistance together with other signs of increased capillary permeability The increased permeability vanished as rapidly as the vasodilator response after infusion ACh and ATP dilated the capacitance vessels but bradykinin histamine and exercise did not It is concluded 1) that all procedures studied increase nutritional blood flow 2) that bradykinin and histamine produce a promptly reversible increase of capillary permeability in the same doses that are needed for dilatation and 3 that none of the substances when infused i a could exactly reproduce the vascular response of exercise

During the past few years several reports have indicated that various vasodilating procedures do not equally affect the different consecutive vascular sections of an organ Thus histamine has been claimed to dilate the arterioles and constrict the veins Haddy 1960) and the dilating action of the nitrites appears to be focused on the capacitance section while hydralazine acts predominantly on the resistance vessels (Ablad and Mellander 1963 With a plethysmographic method combined with direct recording of blood flow Mellander 1960 it is possible to assess quantitatively and simultaneously the effects of a drug on the resistance vessels the capacitance vessels and the capillary filtration coefficient CFC The present work indicates that it may also be possible to reveal increases in capillary permeability

The drugs chosen for this study were acetylcholine (ACh) adenosinetriphosphate (ATP) histamine bradykinin and in a few experiments kallidin The vascular effects of these drugs in an isolated skeletal muscle region were compared with those elicited by exercise because they have been claimed to play a role in producing the hyperemia of exercise

Methods

Experiments were performed on 35 cats weighing 2 5—5 2 kg The animals were anesthetized with chloralose 60—80 mg/kg i v after ether induction and were furnished with a tracheal cannula Heparin 5—10 mg/kg was given i v

P paration The preparation has recently been described in full detail (Kjellmer 1964) The paw was removed at the ankle joint the skin was d vided circularly as h gh up the th gh as possible and dissected free down to the level of the knee jo nt The thigh muscles were div ded by cautery just proximal to the knee leaving the popliteal artery and ve n intact

The ips lateral femoral artery was cannulated and the blood passed through a perfusion pump (S gmamotor model TM 11) to the cannulated popliteal artery The perfusion tubing had three s de tubes the first one for int a arterial infusions — proximal to the pump to ensure complete mixing with the blood the second one distal to the pump for recording of pump pressure and the third one distal to the pump for record ng of the temperature of the perfusing blood which was mainta ned at 36—38 C.

Reco d ng The popliteal vein was cannulated and the blood flow recorded with a photoelectric drop counter attached to a drop chamber filled with s licone oil The outflow level f om the drop chamber could be va ied and its distance above the tissue corresponded to the venous outflow pressure

The calf was placed in a plethysmograph and the skin previously covering the th gh was used as a watertight seal The volume changes of the waterfilled temperature-controlled plethysmograph were recorded with a piston recorder The system c blood pressure was measured f om the contralateral f moral artery with a m rcury manometer All record ngs were made on smoked kymograph paper

Drug infus ns The drugs studied were acetylcholine (Roche) histamine hydrochloride adenosine tripho phate as the disodium salt — ATP (Pabst laboratories) synthetic bradykinin (Sandoz) and synthetic kallid n (Sandoz) both as hyd ochlorides Equal volumes of 0 9 per cent salin and of bradykinin-diluent were infused in control experiments The substances wer dissolved in 0 9 per cent saline in concentrations between 1 and 1000 μg ml The solut ons we e infused into the perfusion tub ng with a constant infusion apparatus at rates varying between 0 04 and 0 30 ml min U ually more than one drug was stud ed in each cat The o der of drug administrations was varied tart ng usually at the lowest doses.

Calcul tions Peripheral resistance was calculated by d vid ng the perfus on pressure (pump p essure m nus venous outflow pressure) with the blood flow in ml (min 100 g) and expressed as peripheral resistance units — PRU The minimum PRU value obtained dur ng infusion was also calculated in per cent of control value

CFC was determined by suddenly raising the venous outflow pressure (usually by 10 mm Hg) This resulted in a sudden increase in volume due to v nous distens on followed by a gradual increase of volume due to net outward cap llary filtrat on CFC was calculated from the filtration slope and expressed a ml (min mm Hg 100 g The 10 mm Hg appl ed to the venous side resulted in almost the same rise of pump pressure and i was th efore assumed that 100 pe cent of the appl ed p essure as transmitted to th capillary level

Results

Effects on blood flow resistance Maximum dilation could be obtained with each of the drugs The vascular resistance dropped from the control level of 20—40 PRU to 5—10 PRU This corresponded to a maximum d latation since even very large addi

[1] The drug was generously supplied by S ndoz A. G. Basel by the courtesy of Dr A Cerletti

TABLE I Doses producing maximal dilatation of resistance vessels

Substance	No of animals	µg/min		µg/(100 g × min)		Molecular weight
		Mean	Range	Mean	Range	
Bradykinin	9	7	1—19	12	1—48	1 160
ATP	7	54	15—150	93	27—278	551
Acetylcholine	11	6	1—11	10	1—24	163
Histamine	9	6	1—13	10	2—27	159

tional amounts of dilating agents or severe exercise did not reduce the peripheral resistance any further The doses needed for producing maximum dilatation are give in Table I together with the molecular weights of the substances Only those experiments are included in this table in which the minimum dose causing maximum response was estimated Although there was considerable variability among animals it is evident that ACh histamine and bradykinin are equally effective when compared on a weight basis while bradykinin is equally effective in only about one tenth of the dose when expressed per mole ATP is the least potent drug both on a weight and molar basis

The dilatation was usually fairly stable during the 5—15 min duration of the infusions but sometimes the vascular resistance returned towards control level despite continued infusion This was seen only rarely with ACh ATP and histamine but occurred in two thirds of the experiments with bradykinin and kallidin

Control infusions of 0 9 per cent saline had no effects Bradykinin diluent was compared with bradykinin in 2 expts It caused some vasodilatation at the highest infusion rates amounting to at most 20—30 per cent of the dilatation obtained with bradykinin It seems possible therefore that a small part of the effect of bradykinin may be ascribed to the solvent

Effects on capacitance vessels The left hand panel of Fig 1 shows the effect of ACh on tissue volume It produced an immediate increase of volume well correlated in time to the decrease of resistance and therefore considered to represent the relaxation of the capacitance vessels This interpretation is corroborated by the findings of Åblad and Mellander (1963) who could correlate this phase of volume gain with an increased regional blood volume as measured with a radio-isotope technique ATP produced the same type of response as ACh with a dilatation of the capacitance vessels the degree of which was related to the extent of dilatation of the resistance vessels By contrast histamine (right hand panel of Fig 1) and also bradykinin (Fig 2) kallidin and exercise produced only a transient initial increase of volume that had passed totally when the perfusion pressure had reached its new level i e no signs of an active change of regional blood volume (see discussion) Fig 3 summarizes the effects of the different drugs and of exercise on the capacitance vessels in relation to the degree of resistance change It appears that neither exercise histamine nor bradykinin (or kallidin) changed the tone of the capacitance vessels significantly even when a maximum dilatation of the resistance vessels was reached while ATP and ACh produced a graded relaxation of the capacitance vessels amounting to at most some 0 2—0 3 ml increase of regional blood volume/100 g muscle

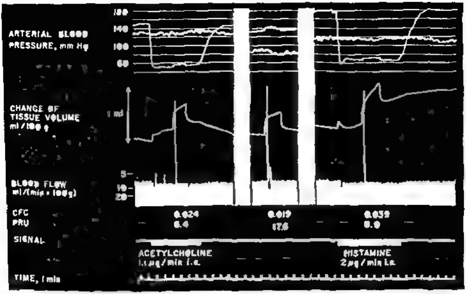

Fig 1 Cat 3 7 kg The effect of acetylcholine and histam ne on perfus on pressure t ssue volume CFC and PRU dur ng constant blood flow In this Fig as in Fig 2 the perfusion pressu e s represented by the thinner lin while the systemic blood pressure gives a thicker and more irregular t acing

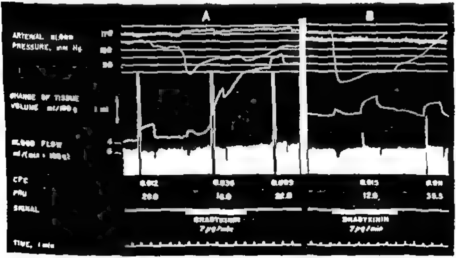

Fig 2 A Cat 4 1 kg B Cat 3 9 kg The ffect of br dyk n n on perfus on p essu e tis ue volume CFC and PRU during nstant blood flow in 2 expts P n l A shows the most common type of response wh re a l g r ase of CFC occur ed togethe th an irreversible sw ell ng f the muscles Pan l B shows the oth type of respons with a m oder te hange of CFC and without edem form tion

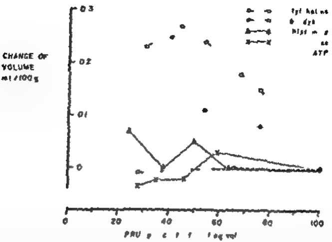

Fig 3 The relationship between increased tissue volume (regional blood volume) and decreased peripheral resistance induced by ACh (42 infusions 14 cats) bradykinin (44 infusions 14 cats) histamine (?8 infusions 10 cats) ATP (33 infusions 8 cats) and exercise (19 exercise periods 8 cats)

Effects on capillary filtration The response of the tissue volume to the drugs infused can be divided into two phases The first phase is represented by the changes reported under Effects on capacitance vessels The second phase is the one that occurs when the dilatation of the resistance vessels has attained a steady state During this period ACh ATP and exercise increased tissue volume only little and after infusion the volume declined to about control level within some minutes (Fig 1 left hand panel)

Histamine and bradykinin on the other hand produced a progressive swelling of the muscles that took place during the whole period of vasodilatation and which persisted for the remaining hours of the experiment These changes occurred in all ten experiments with histamine (Fig 1 right hand panel) and in ten of the experiments with bradykinin (Fig 2 A) In the remaining four bradykinin experiments no such increase of tissue volume during the second phase was obtained despite a profound decrease of blood flow resistance (Fig 2 B) Persistent tissue swelling was seen in two of the four experiments with kallidin

All substances studied caused an increase of the capillary filtration coefficient (CFC) The magnitude of the increase of CFC was related to the degree of dilatation of the resistance vessels although there was a great variability both during the same experiment and particularly between experiments

It was a consistant finding that at equal degrees of dilatation ACh and ATP increased CFC about as much as did exercise whereas bradykinin and especially histamine produced a much larger increase of CFC This is illustrated both in Fig 1 where the effects of ACh and histamine are compared in the same experiment and in Fig 4 where the effects of all the various substances are compared with that of exercise

The large increase of CFC occurring during infusions of histamine and bradykinin were invariably simultaneous with a marked swelling of the tissue (cf Fig 1 and 2)

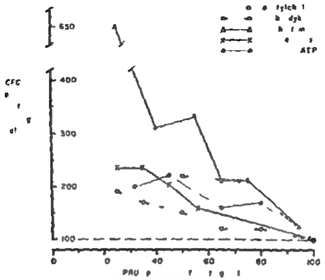

Fig 4 The relatio ish p between increased CFC and decreased peripheral resistance induced by ACh (4? infusions 14 cats) bradykinin (44 infusions 14 cats) histamine (28 infusions 10 cats) ATP (33 infus ons 8 cats) and exercise (19 exercise periods 8 cats)

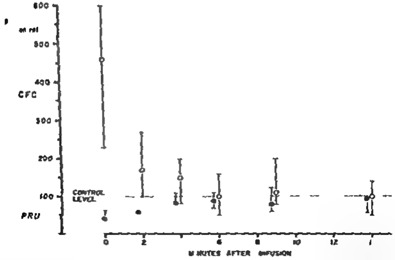

Fig 5 R turn of CFC and PRU after infus ons of h tamine Control level denotes level immed ately before infus ons Values during infusion r presented at ▮ min after infusions 8 expts Values are classed according to the time scale Mean value and range is given for each class F lled symbols PRU hollow symbols CFC

The return of the CFC toward control values was rapid This holds true for exercise for drugs causing changes in tissue volume attributable to capacitance vessel dilatation (ACh ATP) and for drugs causing irreversible tissue swelling throughout their infusion Fig 5 summarizes the time course of recovery of CFC and of PRU after infusions of histamine in 8 cats The earliest interval after which CFC can be estimated reliably is 2—4 min It may be seen that then the CFC had already returned to values close to the control In general recovery of CFC after histamine and bradykinin (Fig 3 A) was correlated closely with recovery of resistance vessel tone

Discussion

The feature common to the substances studied is their ability to decrease flow resistance Since we wanted to compare the composite patterns of vascular response elicited by the drugs with that of exercise the degree of resistance change was chosen as the basis of comparison This was considered to be a physiologically more meaningful type of comparison than comparison on a dose basis particularly since the doses needed showed great variability (cf Table I)

Changes of capillary filtration coefficient (CFC) can be due to changes of the surface area available to filtration to changes of capillary permeability or to a combination of both The changes in CFC occurring during exercise have been shown to depend solely on an increase of perfused capillary surface area (Arturson and Kjellmer 1964)

In the present study capillary permeability was considered to be increased when two conditions were obtained simultaneously 1 CFC should be higher than that due to increased surface area alone i e the CFC value should exceed the CFC recorded during exercise producing a comparable vasodilatation and 2 The volume curve should show sustained increase of tissue volume during the infusion which did not return towards resting volume after termination of the infusion indicating a leakage of protein rich fluid into the tissues

ACh and ATP were found to increase CFC just as much as does exercise i e a 2—2 5 fold increase Furthermore despite the fact that lymph flow was blocked neither of the drugs nor exercise caused any sustained fluid loss into the tissues even when combined with a simultaneous increase in venous pressure As soon as vascular tone and normal venous pressure were re established the volume returned gradually to control level suggesting reabsorption of an essentially protein free fluid from the tissues to the blood stream Hence no signs of increased capillary permeability were present when ACh and ATP were given but these substances like exercise evidently open up previously closed capillaries distributing the increased flow over a larger capillary surface area

On the contrary histamine and in most experiments bradykinin produced not only marked increases of CFC but also a permanent swelling of the tissue suggesting that a fairly protein rich edema was formed These substances therefore fulfilled both the mentioned requirements considered to indicate increased permeability confirming earlier findings (Lewis and Grant 1924 Elliott Horton and Lewis 1960 Sturmer and Berde 1963 It is however quite likely that part of the CFC increase produced by these substances is due to opening of precapillary sphincters making a larger capillary surface area available to blood flow (e g Waldino Hyman and Lenthall 1962)

The CFC values rapidly returned to control values after all infusions This was also true after histamine and bradykinin where the CFC values returned back to pre infusion levels largely at the same rate as vascular tone was re-established (Fig 3 A and 5) It must be concluded that the histamine or bradykinin induced increase of permeability is rapidly reversible which suggests a physiological change rather than a true damage to the capillary walls This is in keeping with the results presented by Miles and Miles (1952) who showed with the dye leakage technique that the increased permeability caused by intradermally injected histamine had largely passed after 10—15 min They also showed that for some hours after histamine the vessels were immune with respect to permeability changes to new doses of histamine This finding could not be repeated in the present study where iterated infusions of histamine produced the same results Moreover the present results suggest that the concentrations of histamine and bradykinin needed to increase capillary permeability by no means are larger than those needed to produce relaxation of vascular smooth muscle but that these two effects appear to run closely in parallel

The capacitance vessels reacted differently to the drugs studied Both ACh and ATP induced a clearcut increase of regional blood volume the degree of which was releated to the response of the resistance vessels (Fig 3) Passive distension of the postcapillary side can be excluded under conditions of constant flow perfusions — the mean capillary pressure staying virtually constant — and therefore this increase of volume must be ascribed to a true dilatation of the capacitance vessels

The capacitance response to bradykinin histamine and exercise differed from that to ACh and ATP Initially there was a small increase of volume which however returned to the resting value as soon as the perfusion pressure had fallen to the new level As this transient volume increase occurred before the pressure drop was fully established it was considered to be merely a passive distension due to the increased pressure head then reaching the capacitance section As soon as the perfusion pressure had fallen so low as to prevent an increased pressure from reaching the postcapillary section there was never any significant increase of volume that could be ascribed to a true dilatation of the capacitance vessels as a response to histamine bradykinin or exercise

The results from the infusions of ACh are in complete accordance with the findings of Ablad and Mellander (1963) who also showed that hydralazine almost exclusively dilated the resistance vessels In this respect hydralazine seems to exert similar effects as histamine and bradykinin to judge from the present experiments The findings that histamine did not relax the capacitance vessels when blood flow resistance was reduced agrees with the results of Glover *et al* (1958) who stated that the high pressure capacity vessels but not the low pressure capacity vessels are dilated by histamine They suggested however that these latter vessels may already have reached their maximal distensibility in the resting warm subject a view which was challenged by Watson (1962) who showed that the distensibility could be further increased during sleep The present results with ACh and ATP also show that the capacitance vessels are not totally devoid of tone even in a denervated preparation though no doubt their tone is low (see also Folkow and Öberg 1961) Haddy (1960) on the other hand who did not record volume changes but measured pressures at various levels of the vascular tree concluded from the pressure changes that histamine dilates the arterioles but actually constricts the veins This would increase capillary pressure and *per se* might according to Haddy be an adequate explanation of the edema produced by

histamine However the fact that the changes in volume were not reversible in the present experiment, strongly suggests that an increase of permeability with protein leakage takes place This would explain part or possibly all of the histamine edema Moreover the present observations concerning the reactions of the venous vessels in terms of capacitance respon■ do not indicate any significant venous constriction However species and tissue difference may exist here Haddy's experiments being performed on skin of dogs the present ones on muscles of cats

When compared to exercise hyperemia it is obvious that the vasodilator response produced by histamine and bradykinin differs with regard to the CFC response and the sustained swelling of the tissue while that of ACh and ATP differs with regard to the capacitance response Thus none of the drugs studied could exactly reproduce the vascular response pattern in the muscles produced by exercise It therefore seems unlikely that any of them should constitute the dominant metabolite producing the exercise hyperemia The possibility cannot however be entirely excluded but then either one of two complementary assumptions have to be made either the substance is released in such a special way that its actions cannot be imitated by i ■ infusions or a combination of substances are released that together yield a response characteristic of that produced by exercise Until one of these additional assumptions has some experimental proof there are no reasons to assume that ACh ATP histamine bradykinin or kallidin play any major role in eliciting exercise hyperemia

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Acta physiol. scand. 1965. 63. 103—110

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Differential Supraspinal Control of Inhibitory and Excitatory Actions from the FRA to Ascending Spinal Pathways

By

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Abstract

Carpenter, D., I. Engberg and A. Lundberg. *Differential supraspinal control of inhibitory and excitatory actions from the FRA to ascending spinal pathways.* Acta physiol. scand. 1965. 63. 103—110. — In the decerebrate state there is an effective tonic inhibition of transmission from the flexor reflex afferents (FRA) to ascending spinal pathways. The release from this tonic control after transverse lesions in the brain stem has been investigated with transmission to ventral spinal pathways. A low pontine lesion releases transmission of inhibitory actions from the FRA whereas a more caudal medullary lesion is required for the release of excitatory action. This parallels what previously has been found with synaptic actions to motoneurones and the differential release of inhibition and excitation from the FRA seems to be a general rule. The results are discussed mainly in relation to the functional significance of the excitatory and inhibitory actions from the FRA to ascending spinal pathways.

Many ascending spinal pathways are influenced from the flexor reflex afferents (FRA). This holds true for at least one subdivision of the dorsal spinocerebellar tract and one of the spinocervical tract (Lundberg and Oscarsson 1960, 1961, Lundberg 1964). Neurones of the ventral spinocerebellar tract (VSCT) are usually inhibited from the FRA but some receive excitation (Oscarsson 1957, 1960, Eccles, Hubbard and Oscarsson 1961, Lundberg and Oscarsson 1961). Neurones of the ventral spinobulbar tract (the bilateral ventral flexor reflex tract, bVFRT) predominantly receive excitation from the FRA (Lundberg and Oscarsson 1962).

There is a parallelism in the supraspinal control of transmission from the FRA to motoneurones and ascending pathways. This is found both for the tonic inhibitory control in the decerebrate state (Eccles and Lundberg 1959, Holmqvist and Lundberg 1959, Holmqvist, Lundberg and Oscarsson 1960a) and for the facilitatory action that

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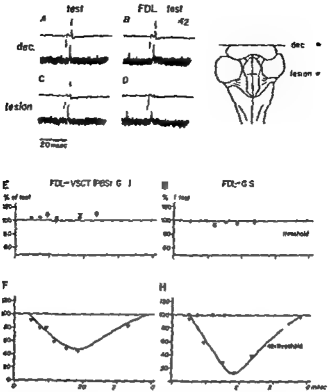

Fig. 1. In this experiment both ventral quadrants were dissected in the lower thoracic region but the dorsal part of the spinal cord was intact. In A–D the upper traces show monosynaptic test reflexes to G.S. evoked and recorded in the nerve to this muscle; lower traces are monosynaptic VSCT discharges recorded in the right ventral quadrant and evoked on stimulation of the left nerves to PBSt and G.S. B and D show the effect of conditioning volleys in high threshold muscle afferents from FDL in the decerebrate state and after the low pontine lesion respectively. The time course of the actions is shown in graphs E–H. In E and G the conditioning stimulus applied to the FDL nerve was 2.8 × threshold while in F and H it was 42 × threshold.

can be evoked from the sensorimotor cortex (Lundberg and Voorhoeve 1962; Lundberg, Norrsell and Voorhoeve 1962; Magni and Oscarsson 1961; Lundberg, Norrsell and Voorhoeve 1963). It has been suggested that the FRA message of the ascending spinal paths may be informative of flexor reflex patterns. This hypothesis requires parallelism in supraspinal control and has been further tested in the present investigation. There is evidence of a differential control from the brain stem of the inhibitory and excitatory FRA paths to motoneurones. A low pontine lesion gives release from the tonic decerebrate control of the inhibitory paths from the FRA to motoneurones whereas release of the excitatory paths only occurs after a more caudal medullary lesion (Holmqvist and Lundberg 1959, 1961; Holmqvist 1961). Similar findings will now be reported with respect to the supraspinal control of inhibitory and excitatory actions from the FRA to ascending pathways. The results will be discussed in relation to the functional significance of excitatory versus inhibitory actions from the FRA on ascending spinal pathways.

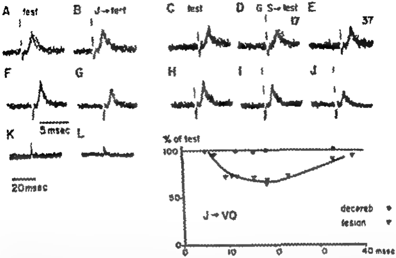

Fig 2 Same experiment as in Fig 1 The test discharges (unconditioned in A F C H) were evoked on stimulation of the left dissected ventral quadrant and recorded from the right dissected ventral quadrant. B and G show the effect of conditioning volleys in the posterior nerve to the knee joint (J) and D E I and J of conditioning volleys in the nerve to G S with strengths indicated in multiple of thresholds A J were obtained with a strobed sweep and the interval between conditioning and testing shown in K and L were taken at the slower speed Records A [illegible] were taken in the decerebrate state F J after the lesion indicated in Fig 1

Methods

The experiments were performed on unanaesthetized cats decerebrated at the intercollicular level The floor of the 4th ventricle was exposed by removal of cerebellum and transverse brain stem lesions were made as described by Holmqvist and Lundberg (1961) The ventral quadrants of the spinal cord were dissected in the lower thoracic region but the dorsal cord where the descending path from the supraspinal brain stem centers is located was left intact (cf Holmqvist *et al* 1960 a) I b volleys from the contralateral gastrocnemius-soleus G S and posterior biceps semitendinosus (PBSt) nerves were used to evoke a test discharge in the VSCT (Oscarsson 1956) Activity in the bVFRT was investigated on stimulation of contralateral or ipsilateral muscle and joint nerves. For testing excitability changes in bVFRT neurones the descending monosynaptic connection described by Holmqvist, Lundberg and Oscarsson (1960 b) was used

Monosynaptic reflexes were recorded in ventral roots or in the peripheral nerves to PBSt and G S The nerves were dissected to permit the placing of both stimulating and recording electrodes on them

Results

Release of inhibitory actions

There was always a parallelism in release from the decerebrate inhibitory control of inhibitory actions from the FRA to motoneurones and to ascending pathways In Fig 1 is compared the effect of conditioning volleys in high threshold muscle afferents on monosynaptic test reflexes upper traces A D and on the monosynaptic VSCT discharge (lower traces A D evoked from I b afferents and recorded in the contralateral spinal half In the decerebrate state there was no inhibition of either the VSCT or the G S nucleus record [illegible] and graphs F and H but after the low pontine lesion

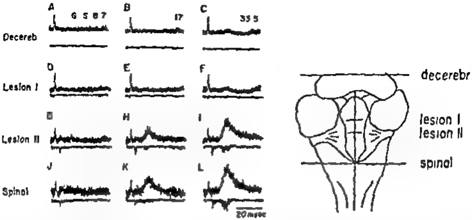

Fig 3 Release of excitatory action from high threshold muscle afferents to ascending pathways and to motoneurones Decerebrate cat with ventral quadrants dissected in the lower thoracic region The left G S nerve was stimulated at strengths indicated in multiples of threshold above each column Recording from the right ventral quadrant (upper traces) and from the S_1 ventral root (lower traces) The initial discharge in the upper traces is the monosynaptic activation of the VSCT from I b afferents Each row of records was taken after the lesion indicated to the left and shown in the right hand diagram

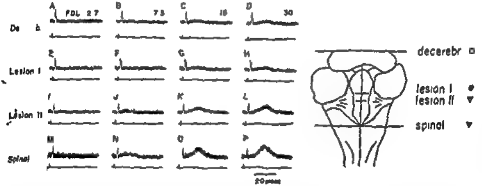

Fig 4 Record ng from right ventral qua lrant (upper traces) and fr m the dorsal r t entry zone n L 7 (lower traces) The left FDL nerve (interosseus nerve remo ed) was stimulated at strengths ind cated above each column of re ords Each row of recor ls was ol ta ned after the lesions indicated to the l ft and shown in the right hand diagram The recor ls shoul l be compared with those of Fig 3 from the same experiment

both discharges were inhibited to approximately the same degree (record D and graphs F and H) The inhibitory action was evoked from high threshold muscle afferents Graphs E and G show that conditioning group I volleys in the nerve from flexor digitorum longus (FDL) had no effect Record D also shows that the conditioning volley did not evoke any late mass discharge i e there is no release of excitatory actions from the FRA

The bilateral ventral flexor reflex tract (bVFRT) is excited from the FRA in the spinal state (Lundberg and Oscarsson 1962) The neurones of this pathway receive a

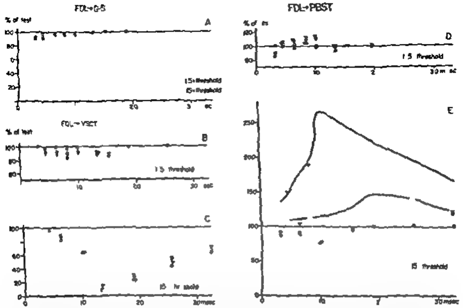

Fig 5 The graphs are from the same experiment as the records of Fig 4 In all curves the conditioning volleys are in the FDL nerve and the strengths are indicated in multiples of threshold strength 1.5 being maximal for group I Monosynaptic reflexes were recorded from the S_1 ventral root and A shows the inhibitory effect in the decerebrate state by a volley in high threshold muscle afferents on the G S monosynaptic test reflex In D and E the monosynaptic test reflex is from PBSt and the curves show the effect of conditioning volleys in the FDL nerve after the lesions indicated in Fig 4 The monosynaptic test discharge in B and C is in the VSCT (stimulation of the PBSt and G S nerves) and the effect of conditioning FDL volleys is shown after each lesion indicated in Fig 4 Observe that the release of inhibitory action to VSCT is almost complete in the decerebrate state but that release of excitation in E only occurs after lesion II

descending monosynaptic excitatory action (Holmqvist Lundberg and Oscarsson 1961) and this monosynaptic test discharge was used to investigate synaptic actions from the FRA on to the bVFRT after a low pontine lesion In Fig 2 there is in the decerebrate state no effect by conditioning volleys in the posterior joint nerve (B) or by volleys in high threshold muscle afferents from G S (E) However after a low pontine lesion the same conditioning volleys markedly inhibit the test discharge (G and J) After transection of the cord there was usually reversal to facilitation (cf Holmqvist *et al* 1960 b) The time course of the inhibition is shown in the graph of Fig ■ The relatively shortlasting action makes postsynaptic inhibition likely

Release of excitatory action from the FRA to ascending pathways

A number of ascending spinal pathways are excited from the FRA However those pathways which are located dorsally cannot be tested because of the fact that the descending paths which are tonically inhibiting transmission from FRA are also located in this part of the cord (Holmqvist and Lundberg 19.0 Holmqvist *et al* 1960 a) Hence the investigation was limited to the release of transmission to the

bVFRT The discharge in this pathway can be recorded in the ventral quadrant and is evoked from the contra and ipsilateral FRA. It was found that a low pontine lesion that can give an almost complete release of inhibition to VSCT usually gives no or very little release of transmission to the bVFRT (cf Fig 1) In Fig 3 the left G S nerve was stimulated at the strengths indicated and discharges were recorded from the right spinal half and from the S_1 ventral root In the decerebrate state there is a very effective control of transmission from high threshold muscle afferents Lesion I hardly gives any release but after lesion II there is a considerable release that somewhat increases after lesion III It is noteworthy that release of transmission from the FRA to the two neuronal systems occurs in parallel

Studies of the release of excitatory and inhibitory effects on conditioned monosynaptic test responses show essentially the same results An experiment which demonstrates this similarity is illustrated in Fig 4 and 5 Fig 4 shows the recording from the ventral spinal quadrant of the mass discharge resulting from conditioning volleys in the FDL nerve at various strength of stimulation before and after the lesions Fig 5 E shows the effect of conditioning volleys in high threshold muscle afferents on flexor monosynaptic reflexes There appears to be a parallelism in the release of excitation from the FRA to the ventral quadrant mass discharge and to the flexor monosynaptic response In both cases the release of facilitation appears after lesion II but increases still further when the animal is made spinal The inhibitory actions to the VSCT were also investigated for group I (Fig 5 B) and the FRA (Fig 5 C) In this experiment there was already in the decerebrate state a marked release of inhibitory actions from high threshold muscle afferents to extensor motoneurones and to the VSCT but there was complete inhibition of transmission to the bVFRT The inhibitory action to the VSCT in contrast to the excitatory action changes very little after the different lesions

Discussion

These experiments have given further evidence of parallelism in supraspinal control of transmission in reflex paths and to ascending pathways The investigation has been concerned with the release from the tonic control that in the decerebrate state completely inhibits transmission of single volleys from the FRA After a low pontine lesion there is release of the inhibitory paths to ascending pathways but for release of the corresponding excitatory paths a more caudal medullary lesion is required The same differential release has previously been found for the inhibitory and excitatory paths from the FRA to motoneurones (Holmqvist and Lundberg 1961 Holmqvist 1961) and therefore seems to be a general rule

It is probable that the descending inhibition is exerted at an interneuronal level and that there are separate descending systems controlling the inhibitory and excitatory reflex paths from the FRA Eccles and Lundberg 1959 Holmqvist and Lundberg 1961 Carpenter *et al* 1963) These descending paths can be assumed to have different chemical specificity one making contact only with interneurones of inhibitory paths and the other only with interneurones of excitatory paths This hypothesis of chemical specificities may as will be discussed below be advantageous in trying to understand the selective significance of the excitatory and the inhibitory actions from the FRA to ascending pathways The hypothesis that the FRA message of ascending pathways is informative of activity in flexor reflex paths requires that the

descending actions on reflex paths is reflected in the ascending activity. The experiments on the supraspinal control of reflex paths to motoneurones have revealed that there may be functional independence of excitatory and inhibitory reflex paths from the FRA (Holmqvist and Lundberg 1961). Hence there is need for separate information to higher centres about events in excitatory and inhibitory reflex paths. If, as proposed above, a descending action is specific for an excitatory or inhibitory reflex path, it follows that information to ascending pathways concerning activity in these reflex paths must be transmitted as excitation and inhibition respectively.

It is postulated that the inhibitory and excitatory actions from the FRA to ascending pathways are informative of inhibitory and excitatory reflex paths respectively. Inhibition can be forwarded as a decrease in frequency of a resting discharge, which may be due to an inherent spinal mechanism (cf. Holmqvist, Lundberg and Oscarsson 1956). However, it is noteworthy that to those ascending pathways that receive marked inhibitory action from the FRA, the VSCT and bVFRT, there are descending excitatory paths that can supply the required background excitation. VSCT neurones receive excitatory action from two descending paths (Magni and Oscarsson 1961) and the bVFRT neurones can be very effectively monosynaptically activated from a pathway *originating in the brain stem* (Holmqvist *et al.* 1960 *b*).

In the spinal state the bVFRT receives *predominantly excitation from the FRA* but evidence for inhibitory action from the FRA to these neurones is found not only after a low pontine lesion (Fig. 2) but also in the spinal state (Lundberg and Oscarsson 1962). Experiments with adequate stimulation have revealed that excitation and inhibition to bVFRT neurones is evoked by the same kind of stimuli (Landgren, Lundberg and Vyklicky, unpublished). There is increasing evidence that the same primary afferents have alternative excitatory and inhibitory paths to bVFRT neurones. This parallels the organization of connections from the FRA to motoneurones (Holmqvist and Lundberg 1961, Holmqvist 1961) and one important function of bVFRT neurones may well be to inform higher centers which one of the alternative paths to motoneurones is active.

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Functional Qualities of Small Blood Vessels in Tissue Injured by Freezing and Thawing

By

OLA BODVAR REITE

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Abstract

REITE, O. B. *Functional qualities of small blood vessels in tissue injured by freezing and thawing.* Acta physiol. scand. 1965. *63*. 111—120. — Rabbit ears were frozen to −10° C and thawed *in vivo*, uniform freezing being secured by vascular occlusion during the freezing procedure. Following thawing and re-establishment of blood flow the ears showed circulatory changes. Hyperemia, edema formation and stasis with subsequent recovery were observed. Studies were performed to test the functional qualities of the small blood vessels of the affected area. The vascular endothelium, the smooth muscles and the sympathetic vasoconstrictor nerve fibres were examined.

The endothelial regenerative power appeared unaltered as revealed by the ingrowth of new capillaries into transparent ear chambers inserted in the injured ears. Intradermal injections of histamine and bradykinin caused a local increase in the exudation of Evan's blue from the frostbitten blood vessels whereas intradermally injected acetylcholine did not. Stimulation of vasoconstrictor nerve fibres or intravascular injections of adrenaline produced vasoconstriction measured as a decrease in blood flow through the previously frozen ear.

It is concluded that the blood vessel walls have retained their functional qualities and that the observed circulatory changes can be induced by endogenous substances liberated or activated consequent to injury.

Tissue damage produced by freezing and thawing is followed by local circulatory changes. The development of hyperemia, edema and stasis is the regular sequence of events (Kreyberg 1946, Crismon and Fuhrman 1947). The severity of the vascular disorders increases when the freezing temperature is lowered (Cohnheim 1873). The degree of the changes produced by frostbite also varies with the duration of cold exposure (Rosenfeld et al. 1949).

As most anatomical structures appear relatively unaltered after freezing and thawing (Rischpler 1900), immediate effects on the tissue must be sought in its functional characteristics. The study of the vascular reactions in frostbite is usually performed

by optical techniques involving transillumination of the injured tissue (Quintanilla, Krusen and Essex 1947, Sullivan and Towle 1957, Reite 1964). Such studies are limited to a description of the resulting changes in the dynamics of circulation and reveal little about the primary site of damage. The observed circulatory disorders may be brought about by vasoactive substances activated or liberated consequent to injury, or by direct damage to the blood vessel walls from freezing and thawing. If the changes are induced by chemical mediators, a functionally responsive vascular bed is required, while a direct damage to the blood vessel walls would be expected to become manifest as failure of the normal responses of blood vessels to stimuli.

This investigation was initiated to provide information about the functional qualities of a frostbitten vascular bed. A standard frostbite was produced in rabbit ears, followed by an examination of blood vessel responses considered as appropriate indicators of vascular viability. The three main functional components of the blood vessel wall, the endothelium, the smooth muscles and the vascular nerves, were tested.

The regenerative power of blood vessel endothelium was studied with the aid of transparent ear chambers. The effect of known permeability increasing substances on the passage of proteinbound dye from blood plasma across the capillary membrane served as a test of endothelial reactivity. Information about the functional qualities of smooth muscles and vascular nerves was evaluated from changes in local blood flow produced by intravascular injections of adrenaline and by vasoconstrictor nerve stimulation.

Material and methods

Experimental animals. Adult lop-eared rabbits weighing 4–6 kg were used for the implantation of transparent ear chambers to study endothelial regeneration.

The permeability test was performed on the ears of adult albino rabbits. Their body weight ranged from 3–4 kg.

Vasomotor function was studied both in albino rabbits and in animals of the lop ear breed. The total number of experimental animals was 19.

Anesthesia. Prior to the actual freezing procedure 0.5–0.6 ml 6 [illegible] sodium pentobarbital (Nembutal, Abbott) per kg b. w. was injected slowly into an ear vein.

Insertion of ear chambers was carried out under local anesthesia produced by 1–? ml 0.5 [illegible] Xylocaine (Astra) injected s.c. This agent was also used for a blockade of vasoconstrictor nerves to the ear by infiltration of a 2 [illegible] solution in the tissue at the base of the ear.

Animals used in the experiments designed to test vasomotor mechanisms were anesthetized by chloralose urethane. A mixture of 4 ml 1 [illegible] chloralose and 3 ml 2[illegible] urethane per kg of body weight was applied, 3/4 of the mixture by i.v. injection and the remaining administered i.p. The rabbits were tracheotomized, and if necessary additional i.p. injections of 3–4 ml 2[illegible] urethane served to maintain anesthesia.

Standard cold injury. A bath for freezing was made by adding crushed ice to a solution of sodium chloride in water under stirring until the temperature of the bath, measured by a mercury thermometer, reached –10 C. To keep the temperature constant at this level small amounts of sodium chloride and crushed ice were added when required.

In the anesthetized rabbit a hypodermic needle was inserted through the skin at the base of the ear on its outer surface. The needle was directed toward the ear tip and pushed forward in the connective tissue layer between skin and cartilage until it reached a point about half the distance from ear base to ear tip. In the next step the needle was pulled back and replaced by a copper-constantan thermocouple. This arrangement was performed to avoid cooling of the thermocouple junction by direct conduction from the surrounding freezing solution through the wire. The temperature was read at a potentiometer (Leeds and Northrup).

A rubber stopper was put into the ear opening and a polyvinyl tubing tied loosely around the ear and the stopper distal to the point where the thermocouple were passed through the

skin under careful precautions not to obstruct the blood flow After wetting the rabbit's ear with water the ear was submerged in the freezing bath up to the level of the polyvinyl tubing The blood flow to the ear could now be shut off simply by tightening the tubing

Within 3—4 min the temperature inside the ear had fallen to — 10 C and was equal to that of the freezing solution The ear remained in the bath for another minute with the temperature maintained at — 10 C Subsequently the freezing bath was taken away and the ear dipped into water of 30—40 C. When withdrawn after 2—3 min the ear had attained a temperature of 30 C The polyvinyl tubing was loosened and the rubber stopper removed

Transparent chamber technique The transparent ear chambers were inserted in the flat portion of the ear at about two-thirds distance from base to tip The operation was performed within the first 24 hours after thawing The chambers used were made in the Institute's own workshop after a modification of Sandison Clark's round table type (Clark *et al* 1930) worked out by Ahlgren Barclay and Ebert (1949) Except for minor deviations the procedure of the latter authors were also followed during installation

The ingrowth of blood vessels and their differentiation were studied at magnifications in the order of 40—200 × A Leitz Ortholux microscope with a Leica camera for photomicrography was utilized The rabbits were examined in their natural sitting position

Permeability test The effect of permeability increasing substances on the blood vessels of the frostbitten rabbit ears was tested in the following way —

Within the first hour of the post thawing period T 1824 (Evans blue) was administered i v (15 mg/kg b w) Subsequently the test preparations were injected intradermally in the frostbitten ear in 0 2 ml of physiological saline Histamine (histamine hydrochloride) and bradykinin (synthetic) served as substances known to affect permeability Acetylcholine (acetylcholine chloride) which is a strong vasodilator with minimal effect on permeability was used as control substance

Nerve stimulation The cervical sympathetic nerve which in the rabbit runs separated from the vagus nerve was dissected free proximal to the superior cervical ganglion The stimulating electrodes were placed on the intact nerve In some animals the nerve was cut and its peripheral end stimulated Dilation of the corresponding pupil indicated that the nerve was being stimulated A Grass stimulator (model S 4 G) was used for the stimulation The characteristics of the stimulation were kept constant (Frequency 15/sec duration 1 msec intensity 6 V)

The experiments with sympathetic vasoconstrictor nerve stimulation were performed at different stages of inflammation from immediately after thawing to 5 days later

Adrenaline injection Adrenaline (1 adrenaline chloride) was given intravenously through a polyethylene catheter introduced in a femoral vein The original solution (0 1) was diluted in physiological saline to a concentration of 10 μg/ml

Blood pressure recording A Statham pressure transducer (model P 23 Gb) together with a Sanborn carrier preamplifier and recorder were used for continuous measurement of systemic arterial blood pressure The pressure transducer was connected with one femoral artery via a polyethylene catheter The entire manometer system was filled with heparinized saline

Measurement of blood flow Changes in local blood flow were measured indirectly in two ways either by changes in available tissue oxygen or by changes in heat flow from the skin

Determination of available oxygen in tissue was performed using polarographic technique (Montgomery 1953) The cathode an uncovered platinum electrode 0 3 mm thick and 5 mm long was inserted in the subcutaneous connective tissue of the distal part of the ear A silver — silver chloride anode was placed under the skin in the scapular region of the rabbit An electronic microampere meter with built in power supply provided the fixed voltage of 0 7 V which was applied between the electrodes The signals from the amperemeter passed through an AC/DC preamplifier and were recorded on the same Sanborn recorder as that used for the blood pressure measurement Prior to nerve stimulation or adrenaline injection the activity of the oxygen electrode was tested by a short arrest of the breathing clamping of the tracheal cannula Electrodes not responding satisfactorily to the asphyxiation were replaced

Variations in heat flow from the skin of the ear were measured by utilizing heat flow disc (Hatfield 1950 The disc consisted of tellurium alloy coated on both faces with copper gauze and had a diameter of 12 mm and a thickness of 1 5 mm Leads from the copper gauze were connected to a microvoltmeter The signal were amplified in a Sanborn DC pre amplifier and recorded During the experiment the heat flow disc was attached to the skin of the ear by thin cellulose tape In order to obtain a stable recording sudden variations in room temperature were avoided

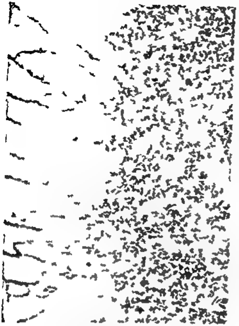

Fig 1 (Left) *Photomicrograph obtained on the tenth day after implantation of an ear chamber in a frostbitten ear. Ingrowth of blood vessels started on the seventh day. The capillary loops have already advanced a distance of about 1 mm along the transparent chamber table. Magnification 28 ×*

Fig 2 (Right) Photomicrograph from transparent chamber in a frostbitten ear 12 days after appearance of the first capillary loops. The beginning of blood vessel differentiation may be seen. The narrow vessel A carries blood to the tissue of the chamber and will later become an artery, while the blood is drained by the vessel V which, like a typical vein, is wider and has a more winding course. Magnification 28 ×

Results

The freezing process became visibly manifest from opacity changes in the thinnest portion of the ear. Subsequently it spread throughout the tissue. In most cases ice crystal formation had started within the first 30 sec of exposure. The appearance of opacity was in all experiments observed before the temperature of the deeper tissue layers of the ear had reached — 6 C. When the ear became opaque, a sudden concomitant hardening of the tissue occurred.

In the first minutes after thawing the exposed ear displayed no signs of injury. After half an hour, however, the ear was warm and reddish and during the following hours an extensive edema developed. On the next day blisters were often present on the inner surface of the ear, and the edematous area, which at first was strictly limited to the frostbitten tissue, had expanded towards the ear base.

Three of the rabbits had already ear chambers with functioning arteries, capillaries and veins in one of their ears at the time of freezing. In these ear chambers aggregates of blood platelets, hemoconcentration and capillary stasis was observed.

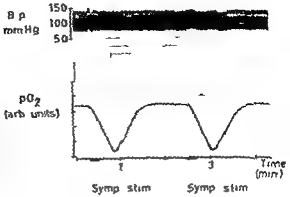

Fig 3 Reduction of pO (available tissue oxygen) in a frostbitten ear as a result of stimulation of the cervical sympathetic nerve on the first day after thawing

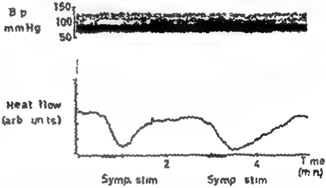

Fig 4 Diminished heat flow from a frostbitten ear caused by stimulation of the cervical sympathetic nerve Frostbite produced two days prior to the experiment

Typical signs of inflammation like redness swelling and increased temperature persisted for 4—5 days and then gradually disappeared In rabbits not used for acute experiments and hence not sacrificed during the inflammatory phase the frostbitten ears survived However in addition to a transient hair loss the affected ears of rabbits followed for several months after injury also showed signs of irreversible tissue damage such as abnormal pigmentation and calcification

Blood vessel regeneration Endothelial sprouts from surrounding vessels grew into the transparent space of the ear chambers where they joined and formed loops The first capillary loops were detectable 5—10 days after the transparent chamber had been inserted (Fig 1) The ingrowth proceeded and the network of endothelial vessels were seen to differentiate into arteries and veins (Fig 2) In 3—4 weeks the formation of new capillaries on the transparent chamber table was completed

Permeability change Following intravenous administration of T 1824 the dye gradually escaped from the blood vessels and was accumulated in the tissue of the frostbitten ear Intradermally injected histamine (2 μg) and bradykinin (7 μg) augmented the extravasation of dye at their local site of application This effect was particularly marked when the test was performed immediately after thawing and before onset

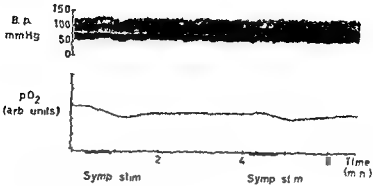

Fig. 5. Stimulation of the cervical sympathetic nerve. The frostbitten ear infiltrated with Xylocain (2 %) at its base. Only a slight decrease in pO

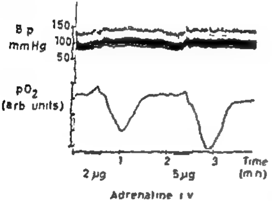

Fig. 6. Decrease of pO in the tissue of a frostbitten ear caused by intravenous injections of adrenalin a few hours after thawing

of extensive edema. Acetylcholine (2 µg) did not induce any corresponding increase in the passage of T 1824 across the capillary membrane. The effect of histamine seemed to be slightly greater than that of bradykinin.

Response to nerve stimulation. Stimulation of the cervical sympathetic nerve usually caused an increase in arterial blood pressure of 4—5 mm Hg. In some experiments this slight rise of blood pressure was absent.

The blood flow through the frostbitten ear showed an immediate and marked decrease upon stimulation of the rabbit's corresponding cervical sympathetic nerve. Stimulation of the contralateral cervical sympathetic nerve had no effect. Results obtained by polarographic determinations of available tissue oxygen (Fig. 3) and by the use of heat flow discs (Fig. 4) agreed closely.

Application of the stimulus on the intact nerve produced the same response as stimulation of the peripheral end of the cut nerve.

During stimulation the ear became paler but the paleness disappeared coincident with the first increase in available tissue oxygen and skin temperature when the stimulation was interrupted.

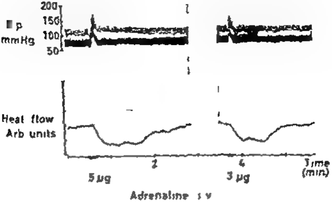

Fig 7 Diminished heat flow from a frostbitten ear following intravenous injections of adrenaline The experiment was performed two days after freezing and thawing of the ear

The blood vessels reacted to nervous stimuli in all experiments performed during the different inflammatory stages

After infiltration at the base of the ear with a local anesthetic agent (2 % Xylocaine) even a prolonged stimulation would produce only a small decrease in available tissue oxygen (Fig 5)

Effects of adrenaline I v injections of adrenaline (1—5 µg) produced a rise in arterial blood pressure of 10—30 mm Hg Within 1—2 min the blood pressure had declined to its normal level

The change in blood flow through the frostbitten ear after injection of adrenaline was similar to that obtained by stimulation of the cervical sympathetic nerve A marked decrease was evident whether polarographic oxygen electrodes (Fig 6) or heat flow discs (Fig 7) were used for the blood flow measurements

Discussion

The rate of ingrowth of endothelial vessels and their differentiation into arteries and veins as observed in the transparent chamber in frostbitten ears do not differ from the findings in unaffected ears reported by Clark *et al* (1931) Similar rates of blood vessel formation in normal rabbit ears are also observed during transparent chamber studies in our laboratory (own observations)

All evidence supports the view that blood capillaries always form from the endothelium of previously existing blood vessels Blood vessel endothelium from the uninjured part of the ear would have to grow a distance of at least 5 cm to reach the transparent ear chamber According to Clark (1936) regenerating capillaries under favourable conditions grow with a rate of 0 1—0 6 mm a day The newly formed capillaries appeared in the transparent chamber 5—10 days after insertion of the chamber in the frostbitten ear This speaks against endothelial ingrowth from blood vessels in the uninjured area It is evident therefore that the freezing procedure used in the

present study does not impair the regenerative power of blood vessel endothelium to any measurable extent

The accumulation of intravascularly injected T 1824 in the tissue of the frostbitten ear indicates an increased capillary permeability An increase in vascular permeability following cold injury is previously demonstrated by several authors using different techniques (Rotnes and Kreyberg 1932 Lange and Boyd 1945 Fuhrman and Crismon 1947) The augmented extravasation of the dye which was observed locally in response to intradermal injections of histamine and bradykinin suggests a *further increase of* permeability at the local site of injection

Apart from their permeability increasing effect histamine and bradykinin also act as powerful vasodilators (Lewis 1927 Elliott Horton and Lewis 1960) Dilatation of the blood vessels of a normal vascular bed does not cause visible leakage of protein bound T 1824 (Miles 1960) In a vascular bed where increased permeability is already established as might occur in frostbitten tissue a local increase in blood flow would be expected to augment extravasation of the dye The inability of intradermally injected acetylcholine to produce increased extravasation of T 1824 shows however that the effect of histamine and bradykinin is not due to their properties as vasodilators Histamine and bradykinin thus seem to increase the permeability of frostbitten blood vessels This shows that the capillary membrane has retained its normal response to these substances and that changes in capillary permeability after frostbite can be mediated by chemical factors

The blood vessel tonus is under humoral and nervous control A vasodilation like that observed in frostbitten tissue might be the result of damage to the sympathetic vasoconstrictor nerve fibres or to the smooth muscle cells of the blood vessel wall However the decrease in blood flow through the frostbitten ear following adrenaline injections and stimulation of vasoconstrictor nerves does not seem to support such an explanation With the systemic arterial blood pressure elevated or remaining constant a diminished blood flow must be ascribed to active vasoconstriction which implies a functionally intact vascular bed

If blood oxygen tension is kept constant and there is no change in the local tissue metabolism the level of available tissue oxygen is expected to vary in direct proportion to nutritional blood flow (Krog 1963) The absence of any change in available ear tissue oxygen during stimulation of the contralateral cervical sympathetic nerve shows that the stimulation does not alter the oxygen tension of the arterial blood It is unlikely that the observed change in available ear tissue oxygen following vasoconstrictor nerve stimulation and intravenous injection of adrenaline is due to an increase in the local metabolic consumption of oxygen Moreover the corresponding decrease in heat flow from the skin of the frostbitten ear and the fact that the ear became paler during stimulation tend to confirm that the decrease in available tissue oxygen actually reflects an active constriction of the frostbitten blood vessels

Stimulation of the cervical sympathetic nerve produced a slight decrease in available tissue oxygen also in ears with their nerve supply blocked (Fig 5) Compared to that found in ears with intact nerve supply (Fig 3) this decrease is very small and may be due to constriction of the arteries central to the ear base or to incomplete blocking The experiment indicates that the response to sympathetic stimulation in ears without nerve block mainly depends on the local frostbitten vascular bed It looks justifiable to conclude that the frostbitten blood vessels show no signs of deterioration in their vasomotor responses

A great number of reports on the patho-physiology of frostbite have appeared in recent years but most investigations deal mainly with therapeutics Vascular viability is only occasionally commented on and the existing data are conflicting

Crismon and Fuhrman (1947) performed microscopic examination of rabbit ears kept for one minute in a freezing bath at —55 C and stated that the vasomotor activity was paralyzed Quintanilla Krusen and Essex (1947) however who studied the small blood vessels in transparent chambers inserted in rabbit ears found that blood vessels previously frozen by application of pieces of solid carbon dioxide directly to the window of the chamber for thirty seconds could still contract as well as the normal ones if the rabbit was frightened or pinched Venous blood flow in the dog leg exposed to temperatures of —25 C to — 40 C for thirty minutes was measured by Hardenbergh and Bamberg (1957) The response to i v injection of adrenaline was an increase in blood flow while i a injections produced a slight decrease As discussed elsewhere (Reite 1964) discrepancies between the different reports may be due to differences in the severity of cold exposure

In the present work the blood vessels responded to all tests applied Vascular endothelium smooth muscles and vasomotor nerves the three main functional components of the blood vessel wall have retained their viability There is therefore no evidence of a specific damage to the blood vessel walls from freezing and thawing *per se* that can account for the observed circulatory changes On the other hand the circulatory changes can well be mediated by endogenous substances activated or liberated as the result of general tissue damage The standard frostbite applied in this work may serve as an experimental model in the study of inflammatory blood vessel responses

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From the Department of Physiological Chemistry University of Lund Sweden

The Metabolism of Fatty Acids in the Rat

II Oleic Acid

By

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Received 13 May 1964

Abstract

Goransson G and T Olivecrona *The metabolism of fatty acids in the rat II Oleic acid* Acta physiol scand 1965 *63* 121—127 — 1 C' Oleic acid and 9 10-H Palmitic acid in rat serum were injected intravenously to fasted and to refed male rats The C and the H³ radioactivity in the tissue lipids was studied at various time intervals thereafter The oleic acid label disappeared more rapidly from the blood FFA than did the palmitic acid label The data indicate that oleic acid is more rapidly oxidized than palmitic acid At short times a larger fraction of the C radioactivity than of the H³ radioactivity of the tissues was in the neutral lipid fraction indicating that oleic acid is incorporated into this fraction to a larger extent than is palmitic acid

A number of differences in the metabolism of individual fatty acids are known to exist Thus some studies indicate that different fatty acids are released and taken up at different rates (Rothlin and Bing 1960 1961) Individual fatty acids are oxidized at different rates (Lossow and Chaikoff 1955) are extracted at different rates (Carlsten *et al* 1962) and are also incorporated into various lipid fractions in different proportions (Dittmer and Hanahan 1959) Furthermore different fatty acids are probably synthesized at different rates and some of the fatty acids may undergo interconversions to other fatty acids (Mead and Howton 1960) All these different metabolic pathways form an interrelated system by which the organism can regulate the fatty acid composition and the concentration of the various tissue lipid fractions

To investigate the possible differences in the metabolism of individual fatty acids we are carrying out a series of studies of the fate of labeled fatty acids after intravenous injection as FFA in rat serum This mode of administration was chosen since it is well known that the plasma FFA represent the transport form of endogenous fatty acids mobilized from the adipose tissue stores and are readily taken up and metabolized by the tissues (Fredrickson and Gordon 1958) A previous study concerned palmitic

acid (Goransson and Olivecrona 1964) This paper presents the results of a study of the metabolism of oleic acid For comparison labeled palmitic acid was simultaneously injected and all data have been calculated as the relative *radioactivity of oleic to* palmitic acid in the tissue lipids

Oleic acid is the main monounsaturated fatty acid in the diet It can be formed in the rat from acetate via desaturation of stearic acid It is present both in the glycerides and in the phospholipids in all tissues but always in a higher proportion in the glycerides The present data show that in relation to palmitic acid the oleic acid disappeared more rapidly from the blood was oxidized more rapidly and was incorporated into the neutral lipids to a larger extent

Methods

The experimental design was to give a group of rats an instantaneous tracer dose of C oleic and H palmitic acid in rat serum Rats were then sacrificed at various time intervals and the C^{14} and H radioactivity in the tissue lipids studied.

1 C^{14} oleic acid (The Radiochemical Centre Amersham England specific activity 21.6 mC/mmole) and 9 10 H palmitic acid (The Radiochemical Centre specific activity 389 mC/mmole) were purified by reversed phase chromatography (Elovson 1964) twice and by subsequent thin layer chromatography to assure that all radioactivity was in free oleic and palmitic acid respectively A solution of the two fatty acids in rat serum was prepared as previously described (Goransson and Olivecrona 1964) 0.5 ml of the serum was injected to each rat This volume contained approximately 0.03 µeq both of the labeled palmitic acid and of the labeled oleic acid

All other experimental procedures were identical with those previously described (Goransson and Olivecrona 1964) The simultaneous determination of C and H radioactivity was performed in a Packard liquid scintillation counter (Olivecrona 1962)

Results

In all the experiments H^3 palmitic acid was injected simultaneously with the C oleic acid The data obtained for the palmitic acid label were in excellent agreement with those published earlier (Göransson and Olivecrona 1964) Therefore the present results have been expressed as the ratio of C to H^3 radioactivity relative to that in the injected fatty acids This approach enables the detection of smaller differences in the metabolism of individual fatty acids than would the comparison of data from *experiments using only single isotopically labeled fatty acids*

Blood radioactivity Table I shows the ratio C /H radioactivity in the blood FFA fraction at short time intervals after the injection The ratios decrease rapidly during the first 2 min in both nutritional states Table II shows the ratio C /H^3 radioactivity in the blood glycerides phospholipids and cholesterol esters The radioactivity at 5 and 10 min was quite low in all fractions and therefore data at these times are not included in the table The glycerides showed ratios of slightly below 1 in the fasted rats and about 1 5 in the refed rats In neither case did the ratio change much with time The phospholipids showed low ratios in both nutritional states with a tendency to rise with time The cholesterol esters showed ratios above one at most times and often considerably above 1

Tissue radioactivity Table III shows the ratio of C H radioactivity in the tissue lipids from the fasted and refed rats respectively The total recovery of radioactivity

TABLE I Ratio C ¹/H rad oactivity in the blood FFA fraction in rats after the i v injection of C -oleic and H palmitic acid in rat serum Each value is the mean of 3 rats and has been normalized to a ratio in the injected fatty ac ds of 1 0

Min	Fasted rats	Refed rats
1	0 71	0 73
2	0 58	0 46
3	0 53	0 40
4	0 51	0 50
5	0 47	0 53

TABLE II Ratio C /H radioactivity in the blood l p ds in rats after the i v injection of C ole c and H palmitic acid in rat serum The ratio in the injected fatty acids was taken as 1 0

	Fasted rats			Refed rats		
Min	Cholesterol esters	Glycerides	Phospho-lipids	Cholesterol esters	Glycerides	Phospho-l pids
20	1 5	0 9	0 1	1 1	1 1	0 2
	0 9	0 9	0 1	1 0	1 6	0 2
40	2 5	0 9	0 2	1 3	1 5	0 2
	1 7	0 8	0 2	—	1 4	0 3
80	1 8	1 0	0 ?	2 3	1 8	0 4
	2 3	—	0 2	2 7	1 6	0 3
160	1 8	0 9	0 2	2 1	1 7	0 4
	1 4	0 8	0 ?	1 8	1 4	0 5
320	1 2	0 7	0 ?	1 7	1 6	0 4
	1 3	0 8	0 2	1 5	1 5	0 5

in lipid soluble form was less for C than for H³ in both nutritional states as shown by the columns total in the rat In most of the indi idual tissues the ratio C /H radioactivity was also below 1 The exceptions were adipose tissue and muscle which showed ratios close to 1 at 5 min and the heart in the refed rats which showed a ratio of 1 4—1 5 at 5 min and then decreased The carcass lipids which presumably represent mainly adipose and muscle tissue showed ratios quite close to 1 initially in both nutritional states In both the fasted and the refed rats this ratio decreased with time

At the early times the ratio was abo e 1 in the liver neutral lipids and below 1 in the liver phosphol pids This was so in both nutritional states In the fasted rats the ratio

TABLE III Ratio $^{14}C/^{3}H$ radioactivity in tissue lipids from rats after the i.v. injection of ^{14}C oleic and ^{3}H palmitic acid in rat serum. The ratio in the injected fatty acids was taken as 1.0. "Total in the rat" represents the ratio in the sum of the radioactivity in all the tissues plus that in the carcass and the blood

Min	Liver Total	Liver NF	Liver PLFA	Adipose tissue Total	Muscle Total	Heart Total	Kidneys Total	Lungs Total	Spleen Total	Carcass Total	Total in the rat
Fasted rats											
5	0.80	1.05	0.43	1.19	0.9[illegible]	0.67	0.48	0.44	0.52	1.06	0.9[illegible]
	0.53	0.95	0.33	0.99	1.0[illegible]	0.68	0.68	0.53	0.42	1.14	0.90
40	0.61	1.02	0.37	1.05	1.54	0.89	0.54	0.50	0.46	1.16	0.95
	0.48	1.00	0.33	1.08	1.03	0.[illegible]8	0.[illegible]1	0.4[illegible]	0.40	1.0[illegible]	0.8[illegible]
320	0.37	0.74	0.25	—	0.87	—	0.59	0.43	0.39	0.81	0.[illegible]1
	0.35	0.92	0.28	1.18	0.7[illegible]	0.41	0.[illegible]1	0.43	0.41	0.97	0.78
Refed rats											
5	0.8[illegible]	1.32	0.53	0.70	1.10	1.4[illegible]	0.9[illegible]	0.[illegible]9	0.[illegible]1	0.99	0.93
	0.81	1.35	0.[illegible]1	0.82	0.[illegible]0	1.[illegible]2	0.80	0.[illegible]1	0.62	0.[illegible]1	0.87
40	0.84	1.[illegible]7	0.57	0.[illegible]1	0.88	1.24	0.75	0.61	0.47	0.89	0.8[illegible]
	0.75	1.[illegible]0	0.40	0.[illegible]8	1.08	1.2[illegible]	0.72	0.[illegible]3	0.5[illegible]	0.[illegible]	0.8[illegible]
320	0.71	1.[illegible]5	0.44	1.01	0.[illegible]8	0.57	0.91	0.80	0.53	0.77	[illegible]
	0.75	1.40	0.[illegible]1	0.[illegible]0	[illegible]	0.72	0.8[illegible]	0.76	0.[illegible]4	0.78	0.[illegible]8

TABLE IV Ratio $^{14}C/^{3}H$ radioactivity in the neutral lipid and the phospholipid fractions of tissue lipids from fasted rats after the i.v. injection of ^{14}C oleic and ^{3}H palmitic acid in rat serum. Mean ± SEM of 3 rats. The ratio in the injected fatty acid was taken as 1.0

Min		Liver	Muscle	Heart	Kidneys	Lungs	Spleen
2	Neutral lipids	1.00 ± 0.03	1.07 ± 0.03	[illegible] ± 0.1[illegible]	0.[illegible]1 ± 0.07	0.4[illegible] ± 0.03	0.4[illegible] ± 0.03
	Phospholipids	0.47 ± [illegible]	0.85 ± 0.03	0.37 ± 0.07	0.44 ± 0.03	0.43 ± 0.0[illegible]	0.40 ± 0.08
320	Neutral lipids	0.61 ± [illegible]	0.63 ± 0.14	0.50 ± 0.10	0.44 ± 0.01	0.43 ± 0.14	0.17 ± 0.01
	Phospholipids	0.37 ± 0.03	0.37 ± 0.03	0.44 ± 0.03	0.[illegible] ± 0.[illegible]	0.43 ± 0.03	0.37 ± 0.03

decreased with time in both the neutral lipids and the phospholipids whereas it remained unchanged in both fractions in the refed rats. In a separate experiment the distribution of radioactivity in the neutral lipid and phospholipid fractions in the other tissues was studied (Table IV). In all tissues the ratio was higher in the neutral lipid fraction than in the phospholipid fraction at two min.

Discussion

In a previous publication the metabolism of palmitic acid after intravenous injection as FFA to rats was reported (Goransson and Olivecrona 1964). This paper describes similar experiments using oleic acid.

To compare the fate of the two acids they were injected simultaneously, the oleic acid labeled with C and the palmitic acid with H^3. Ideally one should be able to study the relation between the metabolism of the two acids by following the fate of the two isotopes. This, however, requires that the two isotopes be representative of the acid in which they were first located. Oleic acid is probably not converted to any other long chain fatty acid to any appreciable extent (Bernhard, Rothlin and Wagner 1958, Dittmer and Hanahan 1959, Elovson 1964 b). Palmitic acid is not converted to other long chain fatty acids to any large extent in fasted rats but in refed rats such conversion is extensive. Thus the H^3 radioactivity in the refed rats represents a mixture of palmitic with smaller fractions of palmitoleic, stearic and C monounsaturated acids (Elovson 1964 c).

The oleic acid label disappeared more rapidly from the circulating blood than the palmitic acid label in both nutritional states. This indicates that the oleic acid in the blood FFA fraction may have a higher fractional turnover rate than the palmitic acid. Fredrickson and Gordon (1958) injected labeled FFA to humans and calculated the fractional turnover rates from the slope of the disappearance curve between 2 and 4 min after the injection. Their data showed similar values for palmitic, linoleic and oleic acid. Laurell (1957) in similar experiments injected palmitic and oleic acid. He did not comment on the relative rate at which the two acids were removed, but inspection of his curves indicates that the palmitic acid was actually removed at a more rapid rate. Uzawa et al. (1964) reported that when labeled oleic and palmitic acid were simultaneously injected intravenously to humans, the labeled oleic acid disappeared more rapidly from the plasma. Although the experiments mentioned above have not consistently shown the same fatty acid to be removed the most rapidly, they clearly indicate that the individual fatty acids in the plasma FFA pool do not have the same fractional turnover rates. This has also been suggested by Rothlin and Bing (1960, 1961) and others on the basis of data on arteriovenous differences of concentration of individual FFA in man.

The recovery of C radioactivity from the whole rats was always less than the recovery of H^3 radioactivity. The loss of lipid soluble C radioactivity indicates oxidation of the fatty acid. The loss of lipid soluble H radioactivity can occur either through oxidation or through desaturation at the 9—10 position. In spite of this added possibility for loss of H^3 radioactivity, the C radioactivity declined faster in both nutritional states. This shows that the oleic acid was oxidized more rapidly than the palmitic acid. As for palmitic acid, the oxidation of oleic acid was more rapid during the first few minutes than later on. However, a continuous loss of radioactivity occurred with both

labels and there was a progressive fall of the ratio C /H radioactivity Thus, the more rapid oxidation of oleic acid was apparent also at later times

The tissue distribution of radioactivity at short times after the injection was different for the two isotopes In most tissues less oleic acid than palmitic acid label was found and only in the heart of the refed rats there was more oleic acid than palmitic acid label This is true in spite of the fact that oleic acid was extracted at a higher rate by the cells This can be explained by oxidation of the fatty acids immediately after their entry into the cell combined with a difference in rate of oxidation of the two fatty acids This explanation is confirmed by the finding that the overall oxidation was rapid and that the oleic acid was oxidized more rapidly than palmitic acid If in addition differences existed in the fractions of the two fatty acids that were taken up by the individual tissues can not be decided from the present data However it has been shown by arteriovenous difference studies in man that the heart preferentially removes oleic acid from the plasma (Rothlin and Bing 1960 1961) and the high oleic acid radioactivity in the hearts of the refed rats is suggestive of a similar mechanism in the rat

In general a higher fraction of the C than of the H^3 radioactivity in the tissue was in the neutral lipid fraction at 2 min (Table IV) This indicates that oleic acid is preferentially incorporated into the neutral lipid fraction and this corresponds to the fatty acid composition of the neutral lipid and the phospholipid fractions (Goransson and Olivecrona 1964) Actually the ratio of C /H radioactivity in the heart and the liver neutral lipids and phospholipids is in direct relation to the chemical amounts of oleic and palmitic acid in these fractions This was not so in the other tissues However the significance of these calculations is difficult to assess since it is known that the tissue lipid fractions do not constitute metabolically homogenous pools (Stein and Shapiro 1959)

When no exogenous fat is present, as in the present experiments the plasma glycerides originate mainly in the liver and the liver and plasma glycerides have a similar fatty acid composition (Goransson and Olivecrona 1964) The present experiments showed that the plasma and liver glycerides also had rather similar ratios of C^{14} to H^3 radioactivity which was to be expected from the considerations above The blood and liver phospholipids did not show identical ratios C H radioactivity but in both fractions this ratio was quite low at all times Karmen Whyte and Goodman (1963) have reported that chylomicron cholesterol ester formation shows marked specificity for oleic acid relative to palmitic stearic and linoleic acids The present data show a similar specificity for oleic acid incorporation relative to palmitic acid for cholesterol ester formation from plasma FFA

This work was supported by grants from the Medical Faculty in Lund and from the U S Public Health Service (He 05307-04 MET

Miss Irene Kalebsson gave skilful technical assistance

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Acta physiol scand. 1965 63 128—132

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Acid-Base Changes in Diving Ducks

By

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Abstract

Andersen, H T P E Hustvedt and A Lovo *Acid base changes in diving ducks* Acta physiol scand. 1965 63 128—132 — The acid base status of ducks has been studied during prolonged diving The pH of the arterial blood has been found to drop roughly 0 4 pH units during submersions lasting for 10—13 min The pCO_2 increases continuously during such a water immersion but the total CO_2 of the blood plasma rises only during the first few minutes and then falls sometimes to sub-resting values This phenomenon has been attributed to the abrupt acidification of the blood.

The large increase in the concentration of hydrogen ions in the blood is only partly caused by CO_2-retention for it has been shown that as much 20 mM l of non volatile acids may be added to the blood plasma during and after diving

Certain vertebrates which depend on lungs for respiratory gas exchange are able to dive for prolonged periods of time (Irving 1939) During submergence a marked retention of carbon dioxide takes place and lack of oxygen causes excessive formation of non volatile acids especially lactic acid Measurements of the arterial pH have shown that the hydrogen ion concentration increases markedly during prolonged submersions (Scholander 1940 Andersen 1959b 1961) These observations indicate that acute acidosis is incurred during submergence but they are insufficient for an evaluation of the acidotic condition

The purpose of the present paper is to describe and discuss the changes observed in the acid base status of diving ducks

Material and methods

Sixteen domestic ducks were studied Their body weight ranged from 14 0 to 21.0 g Diving conditions were simulated by the procedure previously described Andersen 1959a, 1963)

Blood was sampled through a venous catheter i d 0 86 mm inserted 5—10 cm into a wing artery The pH of the blood was measured at 40 C with a Metrohm

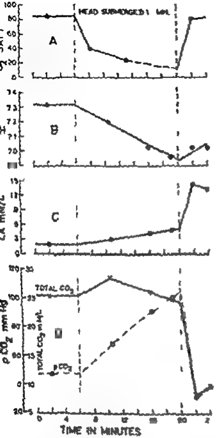

Fig 1 (Left) Changes in arterial oxygen saturation, pH, lactic acid concentration, pCO and total CO before, during and after diving

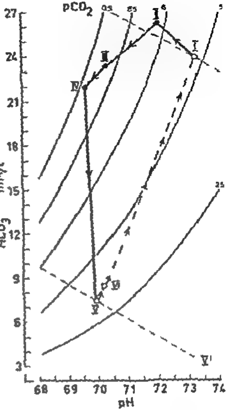

Fig 2 (Right) Pattern of acid base disturbance in the same experiment as shown in Fig 1. Successive points are identified by Roman numbers which relate to points shown in Fig 1 B, C and D. Open symbols: Breathing air. Closed symbols: Submerged

pH meter Model E-300 using a temperature controlled micro glass electrode Model EA 137. The content of lactic acid was determined according to the microdiffusion method of Conway (1962) modified by Scholander and Bradstreet (1962). Blood gases were analyzed in the Scholander Roughton Syringe Analyzer (Roughton and Scholander 1943; Scholander and Roughton 1943). Total plasma carbon dioxide (total CO_2) was determined after anaerobic centrifugation of whole blood at 2200 rpm for 10 min. The partial pressure of carbon dioxide (pCO_2) and the concentration of bicarbonate were calculated from the Henderson Hasselbalch equation using $pK_1 = 6.1$. The buffer line for arterialized duck blood was determined by means of the tonometer technique described by Steen (1963).

Results

Sixteen experiments were carried out The results obtained were very similar and a sample of typical data has been presented in Fig 1 and 2

Oxygen saturation

The arterial oxygen saturation decreased rapidly during the initial 2 min of under water exposure from a resting value of 85 per cent to 40 per cent, and when only half the period of diving had been endured the arterial blood was already less than 25 per cent saturated Extrapolation to the end of the dive indicates an oxygen saturation of about 10—15 per cent just prior to ascent The resting level of oxygen saturation was attained in two minutes or less (Fig 1A)

Hydrogen ions

Measurements of the pH in arterial blood sampled in the prediving period yielded values between 7 32 and 7 48 Fig 1B shows a pH-drop from 7 32 to 6 96 during a submersion lasting for 13 min

Frequently the lowest pH was recorded upon emersion and not at the end of the dive Usually the pH returned to the prediving level in about one hour

Lactic acid

The concentration of lactic acid in the blood rises relatively slowly during diving compared to the steep increase seen upon emergence In the experiment presented in Fig 1C the lactate level rose from a prediving value of 2 0 to 5 1 mM/l after 13 min of submergence During the initial two minutes of the recovery period an additional increase of 8 5 mM/l was observed

Carbon dioxide

Plasma total CO_2 and pCO_2 are shown in Fig 1D It appears that the pCO_2 rose steadily during the submersion whereas the total CO_2 increased only during the initial 4 min and then fell in spite of the continuous rise in pCO_2 At the end of the dive the total CO_2 had fallen below the resting level although the pCO_2 had reached a value more than twice that of the prediving period Both of these parameters drop conspicuously during the early part of the recovery period

Acid base status

Fig 2 illustrates the complete cycle of changes in the acid base status during the same experiment as shown in Fig 1 presented in terms of a pH bicarbonate diagram (Davenport 1958) The concentration of bicarbonate increased in the first part of the dive with increasing pCO_2 and decreasing pH The line I II does not follow the buffer line for arterialized blood but ascends at a steeper slope in accordance with the decrease in oxygen saturation At point II the curve turns around showing a decrease in the bicarbonate concentration although the pCO_2 continues to rise

During the initial stage of the recovery period the pH remained low and the pCO_2 decreased markedly from 100 to 32 mm Hg in approximately 2 min the latter value being 15 mm Hg lower than the resting (see also Fig 1D) The concentration of bicarbonate fell simultaneously from 22 0 to 7 5 mM/l (IV V) The vertical distance I V' shows that roughly 20 5 mM l of non volatile acid had been added to the plasma during the experiment

Discussion

Prolonged submersion asphyxia is accompanied by severe acidosis. The acid base changes observed in ducks during and after extended periods of under water exposure progress in a cyclic pattern when plotted in a pH bicarbonate diagram (Fig. 2). This peculiar development suggests that the acidotic condition is successively respiratory, combined respiratory and non respiratory, and finally purely non respiratory.

During the first minutes of submergence the respiratory acidosis is preponderant. This stage is illustrated by the line I II in Fig. 2. In this period oxygen is consumed at a rapid rate from the lung air which is the main oxygen store in the duck, and the carbon dioxide formed accumulates (Andersen 1959a, b). These changes are closely paralleled in the blood by decreasing arterial saturation (Fig. 1A), increasing pCO_2 and total CO_2 (Fig. 1D), and the pH fell 0.12 pH units (Figs. 1B and 2).

The retention of carbon dioxide forces the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$

from left to right, and the bicarbonate concentration rose (Fig. 2, I II). The line I II ascends at a steeper slope than the normal buffer line in accordance with the continuous decrease in arterial saturation. The latter fell to less than half the resting value during the first couple of minutes of submergence (Fig. 1A). Thus it is reasonable to suggest that anaerobic processes start to operate in the early part of a dive, especially in organs and tissues where the circulation has been slowed down during submergence. Most of the non volatile acids formed are retained in these tissues until submergence (Scholander 1940, Andersen 1961), but a certain amount of fixed acid may leak out into the general circulation.

Initially during diving the increase in blood lactic acid was moderate, but after about 5 min of submergence the resting value had been doubled (Fig. 1C). For the remaining period of the dive the non respiratory component of the acidosis predominated over the respiratory. After 4—5 min of submergence most of the oxygen available had been converted to carbon dioxide, but the pH dropped steadily due to the increase in non volatile acids (Fig. 1B and C). Hydrogen ions in excess are formed when these relatively strong acids dissociate, the reaction (1) is forced from right to left, and carbon dioxide is consequently liberated. This explains how the total CO_2 may decrease while the pCO_2 increases (Fig. 1D). The corresponding fall in the bicarbonate concentration is represented in Fig. 2 by the line II III IV.

Upon emersion the circulatory adjustments from the diving period are abolished and the blood is charged with lactate (Fig. 1C) and other non volatile acids (compare Fig. 2, IV V). These conditions together with a vigorous ventilation of the lungs and airsacs cause a remarkable decrease in the plasma bicarbonate concentration (Fig. 2, IV V). Thus the recovery period is characterized by pure non respiratory acidosis as shown in Fig. 2 by the broken line V I.

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Acta physiol. scand. 1965. 63. 133—140

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Glucuronidation in Rats of Different Ages and Strains

By

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Abstract

J. K. Huttunen and T. A. Miettinen. *Glucuronidation in rats of different ages and strains.* Acta physiol. scand. 1965. 63. 133—140. — Wistar rats excreted more glucuronides into the urine than Sprague Dawley rats both in the basal state and after oral menthol loading. The liver and kidney of the former strain had higher uridine diphosphate glucose dehydrogenase (UDPG dehydrogenase) and β glucuronidase levels than the corresponding organs of the latter strain. Uridine diphosphate glucuronyltransferase (UDP glucuronyltransferase) activity was also higher in the liver of Wistar than of Sprague Dawley rats, the situation being opposite in the kidney. The activity of UDP glucuronyltransferase was higher in the liver and kidney of young rats than of adult rats, the response produced by ethylene diamine tetra acetic acid (EDTA) in the liver being somewhat dependent on the age and sex of the animals. The activities of phosphatases destroying uridine diphosphate glucuronic acid (UDPGA), measured as the release of inorganic phosphate from UDPGA in the presence and absence of EDTA, differed only slightly in the different groups.

Rats of the Sherman strain have been shown to excrete more glucuronic acid but less ascorbic acid into the urine than rats of the Wistar strain (Mossbach, Jackel and King 1950). We have observed that the rate of glucuronidation of 5-hydroxytryptamine occurring in the liver, kidney and intestine is different in rats of the Wistar and Sprague Dawley strains (Airaksinen, Huttunen and Miettinen, to be published). These findings have prompted us to make a more detailed study of the glucuronidation process in these two strains. Since in preliminary studies rats of the Wistar strain were found to excrete more glucuronic acid than Sprague Dawley rats, an attempt was made to elucidate the mechanism underlying this difference on the enzymatic level by an *in vitro* study of the following reactions in the two strains.

(1) UDP-glucose + NAD $\xrightarrow{\text{UDP glucose dehydrogenase}}$ UDP glucuronic acid + NADH$_2$

(2) UDP glucuronic acid + p-nitrophenol $\xrightarrow{\text{UDP glucuronyltransferase}}$ p-nitrophenol glucuronide + UDP

UDP $\xrightarrow{\text{phosphatase (s)}}$ U + 2 P

TABLE 1 Urinary excretion of glucuronic acid in seven week-old rats in the basal state and after oral menthol loading Mean values ± SE

Strain	Basal state (mg/4 hrs)	Menthol loading 20 mg (mg/4 hrs)
Sprague Dawley	0 40 ± 0 06	1 31 ± 0 10
Wistar	0 68 ± 0 08	2 91 ± 0 23

(3) UDP glucuronic acid $\xrightarrow{\text{UDP glucuronic acid pyrophosphatase}}$ glucuronic acid 1 phosphate + UMP

glucuronic acid 1 phosphate $\xrightarrow{\text{phosphatase (s)}}$ glucuronic acid + P_i

UMP $\xrightarrow{\text{phosphatase (s)}}$ U + P

(4) phenolphthalein glucuronide $\xrightarrow{\beta\text{ glucuronidase}}$ phenolphthalein + glucuronic acid

Abbreviations UDP = uridine diphosphate UMP = uridine monophosphate U = uridine P = inorganic phosphate NAD = nicotinamide adenine dinucleotide

Material and methods

Eight male rats of both the Wistar and Sprague Dawley strains of the same age (7 weeks) and weight (125 g) were placed in individual metabolic cages and urine was collected for two 4 hour periods Four ml of water and 1 ml of olive oil were given to each animal in the [illegible]inning of both collections The olive oil given for the latter period contained 20 mg of [illegible]enthol in addition Urinary glucuronic acid was analysed by Dische's carbazole method (1947) as modified by Bitter and Ewins (1961) The animals were killed by decapitation and the liver kidney and intestine were homogenized and analysed for *uridine diphosphate glucose dehydrogenase (UDPG dehydrogenase) uridine diphosphate glucuronyltransferase (UDP glucuronyl transferase) uridine diphosphate glucuronic acid pyrophosphatase (UDPGA pyrophosphatase)* and β *glucuronidase* activities with the methods previously presented in detail (Miettinen and Leskinen 1963) The reactions (2) and (3) were studied in the same incubation mixture by using uridine diphosphate glucuronic acid (UDPGA) concentrations of 0 5 and 1 5 mM in the presence and absence of ethylene diamine tetra acetic acid (EDTA) (8 mM) The inorganic phosphate and p-nitrophenol glucuronide formed were determined The latter measures UDP glucuronyl transferase activity This enzyme is stimulated by EDTA on account of better substrate saturation in the liver (Pogell and Leloir 1961) and especially in the kidney and intestine (Miettinen and Leskinen 1962) of the rat Since EDTA inhibits pyrophosphatase (Pogell and Krisman 1960) the inorganic phosphate released in the presence of EDTA measures the activity [illegible] of the phosphatase (s) splitting the UDP formed by transferase in reaction 2 The in[illegible] inorganic phosphate in the absence of EDTA measures the activities of UDPGA pyrophosphatase and phosphatases of reaction (3) It is apparent that accurate determination of UDPGA pyrophosphatase is not possible by the method used although both the total release of inorganic phosphate in the absence of EDTA and the change caused by EDTA give a fairly good estimate of this enzyme

In addition 5 adult male rats of each strain (age [illegible] months and average weight 270 g) were included No urinary glucuronides were determined The liver kidney and intestine were tested for UDP glucuronyltransferase and UDPGA pyrophosphatase [illegible] as above except that intestinal homogenate was prepared from the isolated mucous membrane instead of from pieces of whole gut

TABLE II UDP-glucose dehydrogenase activity in the liver, kidney and intestine of seven week-old rats. Units (Strominger et al. 1957) per 100 mg of wet tissue. Mean ± SE

Strain	Liver	Kidney	Intestine
Sprague Dawley	44.6 ± 6.6	10.2 ± 1.0	25.2 ± 6.6
Wistar	70.4 ± 9.1	20.6 ± 3.8	28.8 ± 5.2

TABLE III β-glucuronidase activity in the liver, kidney and intestine of seven week-old rats. Fishman units (Fishman 1957) per 100 mg of wet tissue. Mean values ± SE

Strain	Liver	Kidney	Intestine
Sprague Dawley	582 ± 121	174 ± 5	215 ± 13
Wistar	949 ± 29	191 ± 4	174 ± 13

TABLE IV Glucuronidation of p-nitrophenol in the liver of seven week (eight animals) and five month (five animals) old Sprague Dawley and Wistar rats. Mean values ± SE

The assay mixture was either 0.5 or 1.5 mM for UDPGA, 0.5 mM for p-nitrophenol and contained 50 μl (16.7 mg of wet tissue) of 2 000 g supernatant of liver homogenate in a final volume of 0.1 ml in 0.125 M triethanolamine buffer

Concentration (mM) of incubation mixture for		μmoles × 10⁻³ of p-nitrophenol glucuronide formed per 100 mg of wet liver per 30 min			
		Wistar rats		Sprague Dawley rats	
EDTA	UDPGA	Young	Adult	Young	Adult
0	0.5	55 ± 5	41 ± 3	44 ± 4	29 ± 2
20	0.5	49 ± 3	60 ± 4	57 ± 4	54 ± [illegible]
0	1.5	120 ± 8	80 ± 3	85 ± 5	67 ± 6
20	1.5	75 ± 5	8 ± 3	70 ± 5	56 ± 5

Results

As shown in Table I Wistar rats excrete more glucuronic acid into the urine than Sprague Dawley rats both in the basal state and after oral menthol loading.

The activities of *UDPG dehydrogenase* and *β glucuronidase* (Table II and III) were higher in the liver and kidney of the Wistar rats.

UDP glucuronyltransferase activity (Table IV and Fig. 1) was likewise higher in the liver of the Wistar strain in both age groups, adult rats having a lower activity than

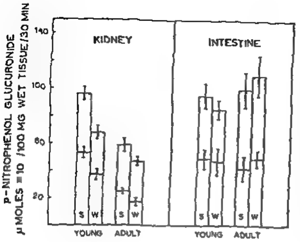

Fig 1 Renal and intestinal glucuronidation of *p*-nitrophenol (mean values ± SE) of the same animals as in Table IV UDP glucuronic acid concentration 1 5 mM Incubation mixture otherwise the same Values for the intestine of adult animals obtained from the 2 000 g supernatant of isolated mucous membrane homogenate Total bar indicates conjugation in the presence and white bar in the absence of EDTA S = Sprague Dawley W = Wistar

young rats In the kidney in contrast to the liver glucuronidation was higher in the Sprague Dawley than in the Wistar rats but even here was lower in adult than in young animals Intestinal conjugation was the same in both strains

Hepatic UDP glucuronyltransferase was activated by EDTA in adult rats in the presence of the low UDPGA concentration but inhibited in young animals in the presence of the high UDPGA concentration Renal and intestinal glucuronidation was strongly stimulated by EDTA irrespective of the concentration of UDPGA This activation was relatively higher however in experiments with the low than with the high UDPGA concentration It is apparent that the UDP glucuronyltransferase assay employed in the present study gives more reliable results in the presence than in the absence of EDTA in the kidney and intestine whereas this is not always the case in the liver

A threefold increase of UDPGA concentration from 0 5 to 1 5 mM in the incubation mixture produced on the average a twofold increase in *p* nitrophenol glucuronidation in the liver and a somewhat greater increase in the kidney and especially in the intestine In the presence of EDTA this increase was less marked and was insignificant in the liver of adult animals

Some experiments with female Wistar rats suggested that EDTA inhibits hepatic UDP glucuronyltransferase activity irrespective of the age of the animals and that as in male rats hepatic and renal glucuronidation is higher in young animals than in adults Further renal conjugation appeared to be higher than in male rats

The release of inorganic phosphate (Fig 2 and 3) which was used to measure *UDPGA pyrophosphatase* increased 2 1 2 3 and 2 8-fold in the liver kidney and intestine respectively when UDPGA concentration increased threefold The activity in the liver was the same in both strains but tended to be higher in adult than in young Wistar rats Only very small amounts of phosphate were released in EDTA experiments irrespective of UDPGA concentration This indicates that EDTA inhibits not only hepatic UDPGA pyrophosphatase but apparently also the phosphatase (s) splitting the UDP formed by transferase Renal UDPGA pyrophosphatase activity was like that of UDP glucuronyltransferase somewhat lower in Wistar than in Sprague-

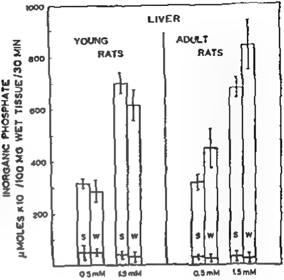

Fig 2 The release of inorganic phosphate (mean values ± SE) from UDPGA in the same incubation as in Table IV Total bar indicates values obtained in the absence and shaded bar in the presence of EDTA Concentration of UDPGA on the abscissa S = Sprague Dawley W = Wistar

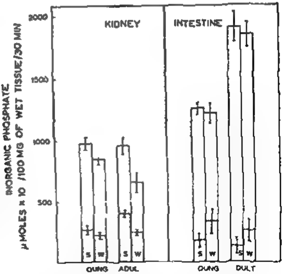

Fig 3 The release of inorganic phosphate (mean values ± SE) from UDPGA in the same incubation mixture as in Fig 1 Total bar indicates values obtained in the absence of EDTA and shaded bar those obtained in the presence of EDTA S = Sprague Dawley W = Wistar

Dawley rats Release of phosphate was markedly inhibited by EDTA especially in young animals The amount of inorganic phosphate released in the presence of EDTA was higher in the kidney than could be expected to be released from the UDP formed by transferase UDPGA pyrophosphatase activity was the same in intestinal preparations of both strains the activity being considerably higher in the mucosal homogenate Again a strong EDTA inhibition was observable

Discussion

It is well known that drug metabolism varies mainly quantitatively between strains of the same species of animals. Good examples of this are the half time of antipyrine in various rat strains (Quinn, Axelrod and Brodie 1958) and the effect of hexobarbital on different mouse strains (Jay 1955). The age (Fouts 1962), sex (Axelrod 1954, Davison 1955, Quinn *et al.* 1958, Inscoe and Axelrod 1960) and nutritional state (Fouts 1962, Miettinen and Leskinen 1963) are factors affecting drug metabolism even within the same strain. Newborn mammals have a poorly developed glucuronidation capacity (Hartiala and Pulkkinen 1955), mainly owing to low UDPG dehydrogenase and UDP glucuronyltransferase activities in the liver (Dutton and Greig 1957). After birth the level of these enzymes increases rapidly to adult values (Brown, Zuelzer and Burnett 1958, Dutton 1959). The behaviour of UDP glucuronyltransferase in the present study further suggests that glucuronidation is highest in young rats, followed by a decrease with advancing age in both sexes. No conclusion can be drawn from the data as to whether this is due to sexual maturation or to aging of the animals. Hartiala and Pulkkinen observed a significant decrease in the glucuronidation capacity when the liver and intestine of 1 month-old Wistar rats were compared with the corresponding organs of 4 month old rats, but they could not detect any difference in the level of glucuronyltransferase (personal communication 1964). The microsomal glucuronidation of o-aminophenol in the liver is higher in male than in female rats and is stimulated by testosterone in the latter and depressed by oestradiol in the former animals (Inscoe and Axelrod 1960). Some of the steroid hormones are known to inhibit transferase activity in vitro (Hsia, Riabov and Dowben 1963). The slightly different response of glucuronidation in the liver of the adult male animals to EDTA and high renal conjugation in female rats are apparent sex differences recorded in the present study.

As far as glucuronidation is concerned, Gunn rats form the most extreme variant thin rat strains. These animals are unable to produce bilirubin glucuronide and excrete even other glucuronides in small amounts owing to low UDP glucuronyltransferase activity (Schmid *et al.* 1958). As already mentioned, the glucuronidation process takes place more effectively in Sherman than in Wistar rats, if judged on the basis of urinary glucuronic acid excretion (Mossbach, Jackel and King 1950). According to the present study the latter strain, on the other hand, possesses a better capacity to produce urinary glucuronides than the Sprague Dawley strain. An explanation on the enzymatic level seems possible, since in the liver both UDPG dehydrogenase and UDP glucuronyltransferase activities are higher in the former than in the latter strain. High renal transferase activity in Sprague Dawley rats may have an opposite action but evidently plays a minor role in glucuronide excretion, since UDPGA production is limited owing to low UDPG dehydrogenase activity and since the size of the kidney is small compared with that of the liver. UDPGA pyrophosphatase activity, as indicated either by release of inorganic phosphate from UDPGA or by the effect of EDTA on it, was the same in the liver and intestine and only slightly different in the kidney of the two strains. Nothing is known of the significance of this enzyme or of β-glucuronidase in glucuronidation in vivo.

Pogell and Leloir (1961) pointed out that hepatic microsomal UDP glucuronyltransferase is apparently both activated and inhibited by EDTA. The former effect was considered to be due to a better substrate saturation on account of UDPGA pyrophosphatase inhibition. Stevenson and Dutton (1962) reported that EDTA had

no effect on intestinal glucuronidation in the guinea pig. Nor had this compound any influence on the activity of solubilized purified glucuronyltransferase provided that no Mg or Ca was present (Isselbacher, Chrabas and Quinn 1962). On the other hand under certain circumstances EDTA can solubilize transferase, so leading to enhanced conjugation (Halac and Bonevardi 1963). Activation, moderate in the liver and more prominent in the kidney and intestine, was observed by us in normal rats (Miettinen and Leskinen 1962, 1963) but not in the liver of nephrotic or thyroxine-treated rats despite marked inhibition of UDPGA pyrophosphatase (unpublished data). In the present study an age-dependent increase in the enhancing effect of EDTA was observed in the livers of male rats but the most prominent enhancing effect was seen in the kidney and intestine of all groups. An inhibiting action clearly demonstrable in the livers of female and young male rats may correspondingly decrease, but may be expected to be detectable in the kidney and intestine as well if sufficiently high UDPGA concentrations are used. On the other hand EDTA produced a remarkable activation of 5-hydroxytryptamine glucuronidation even in the liver at high substrate concentration (Airaksinen, Huttunen and Miettinen, to be published). This was considered to be due chiefly to conversation of labile 5-hydroxytryptamine glucuronide by EDTA. EDTA may similarly inhibit the deconjugation of other glucuronides. Evidence for this has previously been presented for phenolphthalein glucuronide (Miettinen and Leskinen 1963). EDTA thus seems to have at least the following effects on glucuronidation *in vitro*: *1.* It inhibits UDPGA pyrophosphatase, leading via better substrate saturation to increased glucuronidation (Pogell and Leloir 1961). *2.* Protects certain conjugates against deconjugation. *3.* Increases glucuronidation by solubilizing UDP glucuronyltransferase (Halac and Bonevardi 1963). *4.* Counteracts the inhibitory effect of Ca and Mg on transferase (Isselbacher, Chrabas and Quinn 1962). *5.* Inhibits transferase under certain circumstances (cf. Pogell and Leloir 1961).

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From the Institute of Biological Chemistry University of Copenhagen Denmark

Relationship between Osmotic Reactions and Active Sodium Transport in the Frog Skin Epithelium

By

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Abstract

Ussing H H *Relationship between osmotic reactions and active sodium transport in the frog skin epithelium* Acta physiol scand 1965 *63* 141—155 — The osmotic behaviour of the skin epithelium of the brown frog (R temp) has been investigated by microscopic measurement of thickness under different experimental conditions Simultaneous measurements of skin potential and short-circuit current were made Epithelial swelling was induced by hypotonicity of the inside but not the outside bathing solution whereas shrinkage was brought about by hypertonicity of either inside or outside bathing solutions although the tonicity of the inside bathing solution dominates the osmotic reponse Shrinkage can also be induced by lowering the potassium concentration of the inside solution. Complex shrinkage and swelling reactions are produced by addition of diffusible substances like urea to either bathing solution In practically every instance shrinkage leads to inhibition of the short-circuit current (and thus of the active sodium transport) whereas swelling of the epithelium gives rise to increased active sodium transport The skin potential is related to swelling and shrinkage in a more involved fashion In general hypertonicity of the outside bathing solution leads to a violent drop in skin potential The observations indicate that hypertonicity of the outside solution increases the permeability of the outer epithelial boundary to passively diffusing electrolytes and to water

In a previous study (MacRobbie and Ussing 1961) the osmotic behaviour of the frog skin epithelium was investigated by microscopic measurements of its thickness under different experimental conditions The setup permitted simultaneous measurements of epithelial thickness and either skin potential or short-circuit current but whereas the osmotic reactions were studied in considerable detail only cursory observations of the electrical parameters were mentioned This paper deals with the relationship between volume changes and the electrical parameters potential difference short-circuit current and d c resistance In the experiments described below the epithelium of the skin of Rana temporaria have been induced to undergo both swelling and shrinkage by variations in the composition of inside as well as outside bathing solutions The results showed that no matter how the volume changes were brought about, there usually was

TABLE I Ion concentrations in the Ringer's solutions used (mM)

	Chloride Ringer	Sulphate Ringer	Gluconate Ringer	K free Ringer
Na	113 5	113 5	113 5	115 4
K	1 88	1 88	1 88	0
Ca	1 08	1 08	1 08	1 08
Cl^-	115	0	0	115
HCO_3^-	2 4	2 4	2 4	2 4
$SO_4^=$	0	57 5	0	0
Gluconate ion	0	0	115	0

for each individual skin a close correlation between the epithelial volume and the rate of active sodium transport as measured by the short-circuit current Also the skin potential and the d c resistance underwent dramatic changes in association with the osmotic responses

Methods

The experimental setup was that described by MacRobbie and Ussing (1961) The thickness of the epithelium was measured microscopically with a water immersion system (objective Leitz 75 x eyepiece 12 5) The measurement was performed by focussing alternately upon fixed point on the surface of the epithelium and one just below the epithelium The distance read on the fine adjustment screw of the microscope The electrical circuits used for short circuiting or potential readings during the experiments were those described by Ussing and Windhager (1964) The circuits permit automatic recording of either potential or short-circuit current and the voltage can be clamped at any desired potential level The exposed area of skin was in all experiments 7 0 cm and the short circuit currents given in the figures refer to that area The principle for the automatic short-circuiter was that developed by Mullins (1958)

Since sudden increases in ingoing current may elicit potential transients (Finkelstein 1961 Ussing and Windhager 1964) resistance measurements were routinely obtained from the ratio of the increment in potential associated with a sudden decrement in ingoing current (see Ussing and Windhager 1964)

Results

SWELLING DUE TO HYPOTONICITY

a Sulphate Ringer

Fig 1 shows an experiment where initially both sides of the skin are bathed with sulphate Ringer The potential is recorded continuously whereas thickness of the epithelium and the short-circuit current (and d c resistance) are determined at 5 min intervals At the first vertical line the sulphate Ringer flushing the inside is diluted to half strength with distilled water There is a rapid swelling of the epithelium from 46 to a maximum of 61 μ which is reached after 20 min whereafter there is a slow decline When at the second vertical line the outside bathing solution is diluted to half strength there is no significant change in epithelial thickness Neither does the thickness change when the outside medium is again made ordinary sulphate Ringer at the third vertical

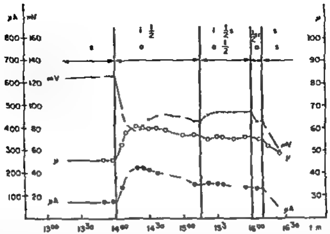

Fig 1 Effect of hypotonicity on epithelial thickness (μ) short-circuit current (μA) and skin potential (mV) (Rana temporaria) Exposed area 7 cm Abscissa Time each division = half an hour Both bathing solutions initially sulphate Ringer (sr) At first vertical line inside solution diluted with an equal volume of distilled water (½ sr) At second vertical line both inside and outside bathing solution are made half sulphate Ringer At third vertical line the outside bathing solution is again made full sulphate Ringer whereas the inside solution remains half sulphate Ringer At fourth vertical line both bathing solutions are made sulphate Ringer

line When however the inside solution is changed from half sulphate Ringer to full sulphate Ringer the volume drops rapidly If we now turn to the short-circuit current it is seen that it undergoes variations which in almost every respect follow those of the epithelial thickness On dilution of the inside solution the short-circuit current starts going up and it reaches its maximum at the same time as does the thickness The changes of the outside solution to half strength sulphate Ringer and back again have no effect on the short circuit current whereas the change to full strength sulphate Ringer on the inside brings about a drop in current *pari passu* with the thickness The changes of the potential are more involved On dilution of the inside solution the potential drops rapidly from 126 mV to reach a minimum after 20 min just at the time when the volume reached its maximum During the slight decline in volume it first increases to 91 mV and then starts on a slow decline The latter is turned into another increase when the outside medium is diluted to half strength A new potential plateau at 94 mV is reached but when the outside medium is changed to full sulphate Ringer the potential starts dropping again and the drop continues when the inside solution is made full sulphate Ringer The d c resistance finally is very high initially (11 500 Ω cm²) and drops sharply to 2 300 Ω cm at the time of the maximum swelling of the epithelium From then on it increases steadily virtually unaffected by the changes in the outside medium but increases sharply to nearly its original value after the change of the inside medium from half to full sulphate Ringer Thus both the short-circuit current and the d c resistance seem very closely correlated to the epithelial volume whereas the potential seems to be a more involved function of the changes in bathing solutions In view of the fact that sulphate is known to penetrate the skin exceedingly slowly and that other ions including potassium hardly contribute to the conductance

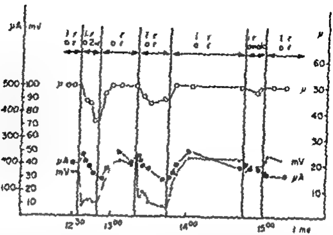

Fig. 4. Dependence of response to hypertonicity of outside solution upon the nature of the osmotically active agent. Epithelial thickness (μ), skin potential (mV) and short circuit current (μA) are recorded. Exposed area 7 cm². Ordinate: time. Inside bathing solution is chloride Ringer throughout. Outside solution first chloride Ringer (o r) then double strength Ringer (o 2 r) back to chloride Ringer (o r) then chloride Ringer made hypertonic by addition of 230 mmoles of urea per liter (o r + u) again chloride Ringer, then chloride Ringer made hypertonic by addition of 230 mmoles ethylalcohol per liter (r alc) and finally chloride Ringer.

At the third vertical line the hypertonic solution on the inside is replaced by sulphate Ringer. The epithelium swells from 35 μ to 48 μ and the short circuit current increases 35 μA to 145 μA. The potential first drops to 11 mV and then returns to 16 mV and stabilizes there. The net effect on the resistance is a pronounced drop.

At the fourth vertical line, finally, the hypertonic solution on the outside is also replaced by ordinary sulphate Ringer. There is a slight but significant swelling and the current as well as the potential both increase somewhat. The experiment shows firstly that the epithelial volume and the short-circuit current are closely correlated and secondly that hypertonicity on the outside of the skin has little effect on thickness and short-circuit current but reduces the potential and the d.c. skin resistance substantially.

b. Chloride Ringer made hypertonic (on the outside) with various substances

It was demonstrated by MacRobbie and Ussing (1961) that there is virtually no volume response to dilution of the outside medium. This conclusion is also borne out by the material presented above (see Figs. 1 and 2). If, however, the outside medium is made hypertonic a more or less pronounced shrinkage is seen. The phenomenon is illustrated in Fig. 4. Initially, both sides of the skin are bathed with chloride Ringer. Potential is recorded continuously whereas thickness and short-circuit current are recorded at about five minutes interval. At the first vertical line doublestrength Ringer replaces the ordinary Ringer in the outside compartment. The epithelium shrinks from 50 μ to 37 μ. The current first rises a little and then drops sharply. The potential drops immediately after the change of solution from 34 to 10 mV. The resistance initially drops from about 1 150 Ω cm² to 250 Ω cm² and then increases somewhat.

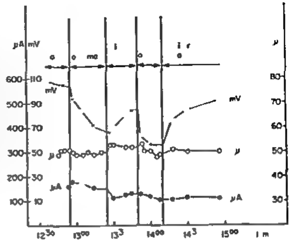

Fig 5 Lack of short-circuit current (μA) response despite good potential (mV) response to hypertonicity of outside solution in skin which gives only minute response of epithelial thickness (μ) Exposed area of skin 7 cm² ordinate, time Inside bathing solution chlorideRinger throughout Outside solution first chloride Ringer (o r) then chloride Ringer made hypertonic by addition of 230 mmole of mannitoles per liter (o r + man) then chloride Ringer (o r) chloride Ringer made hypertonic by addition of 230 mmoles of urea per liter (o r + u) and finally chloride Ringer

At the second vertical line ordinary Ringer is reintroduced at the outside of the skin and volume as well as short-circuit current potential and resistance all go up to slightly more than control values The current thereafter tends to decline again

At the third vertical line the Ringer on the outside is made double isotonic by addition of urea The epithelium again shrinks but only half as much as with NaCl There is a slight hump in the current curve but otherwise it continues its slow decline The potential drops sharply from 45 mV to 14 mV rises a little and again drops slowly during the remaining part of the urea period The resistance undergoes a steep drop from 1 350 Ω cm² to 450 Ω cm² then increases to 700 Ω cm² and decreases again during the rest of the period

At the fourth vertical line ordinary Ringer replaces the hypertonic solution and the volume returns to the control value So do current potential and resistance After a pause during which the volume and the potential stay constant and the short-circuit current drops (and the resistance increases) steadily one per cent ethylalcohol is added to the outside solution. The volume is hardly affected and the current shows only a slight hump but the potential is reduced perceptibly and so is the resistance This effect is reversed when alcohol free Ringer is reintroduced The experiment shows that hypertonicity on the outside gives rise to shrinkage which seems to be less pronounced the more diffusible the osmotically active agent is Furthermore there is a sharp drop in potential and a drop in short-circuit current which is also smaller the more diffusible the agent is

Whereas all skins shrink very substantially when the inside solution is made hypertonic they vary appreciably as to the extent of shrinkage when the outside solution is made hypertonic Fig 5 shows results with one of the least sensitive skins Initially both sides were bathed with chloride Ringer At the first line the outside was made double isotonic by addition of mannitol The shrinkage is barely perceptible and although there is a drop in current the effect is hardly significant The potential on the other hand drops from 95 mV to 66 mV after 30 minutes Thus the skin resistance has dropped from 4 160 Ω cm² to 3 090 Ω cm² When, at the second line the hypertonic outside solution

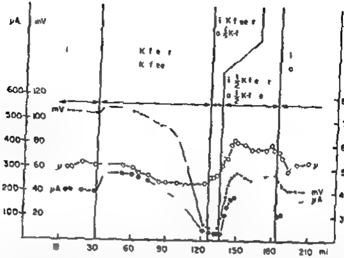

Fig 6 Effect of potassium free Ringer and half strength potassium free Ringer on skin potential (mV) short circuit current (μA) and epithelial thickness of skin of Rana temporaria Exposed area 7 cm Abscissa time in minutes Both bathing solutions initially chloride Ringer At first vertical line both bathing solutions changed to K free Ringer At second vertical line the potassium free Ringer on the outside diluted with distilled water to half strength (½ K free r) At third vertical line the same is done with the inside solution At fourth vertical line ordinary chloride Ringer is reintroduced on both sides of the skin

s replaced by Ringer there is a slight swelling (by 3 μ) and the current shows a slight v to increase The potential rises rapidly and is still increasing when at the rtical line the outside solution is made double isotonic by addition of urea The shrinkage is slight (4 μ) and the current is unaltered or perhaps slightly inhibited The potential however drops very sharply When urea is removed there is a hardly perceptible swelling no significant change in current but a rapid recovery of the potential The experiment shows that when the swelling is slight the changes in current are also slight and furthermore that hypertonicity on the outside reduces the potential and the skin resistance even in cases where the current response is nil This strongly suggests *that the drop in potential is due to an increased shunt rather than a reduced electromotive force of the sodium transport battery*

EFFECT OF SHRINKAGE DUE TO LOSS OF KCl

According to Koefoed Johnsen and Ussing (1958) and MacRobbie and Ussing (1961) the inward facing side of the epithelium is permeable to Cl as well as to K Consequently *if a skin in equilibrium with ordinary Ringer is placed in solutions lacking potassium chloride or both KCl must be lost from the epithelium* and provided the solutions do *not* contain other ions which can enter as fast as KCl is lost a shrinkage should ensue Fig 6 shows an example of this phenomenon At the outset both sides are bathed with chloride-Ringer At the first vertical line the Ringer on both sides is replaced by K *free* Ringer The thickness of the epithelium drops slowly by about 10 μ Potential and current both undergo a sharp rise in the K free medium in accord with the hypoth of Koefoed Johnsen and Ussing (1958) that the diffusion potential for K towards

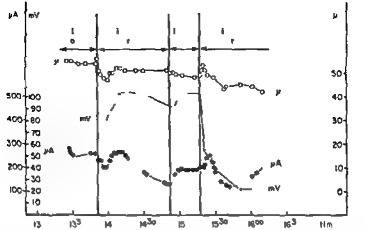

Fig 7 Effects of urea added to the solut ons bath ng the inside and outside of the isolated frog skin upon short-circuit current (μA) skin potential (mV) and ep thel al th ckness (μ) Exposed area 7 cm² Abscissa time Both bathing solutions initially chloride Ringer At first vert cal line the inside solut on is made hypertonic (i r + u) by addition of urea (230 mmoles per liter) At second vertical li e also the outs de solution is made hypertonic with urea At third vertical line ord nary chloride Ringer is re ntroduced on the nside of the skin

inside provides a significant part of the electromotive force of the skin With time how ever both current and potential decline and after 90 min both have virtually vanished Then both the inside and outside bathing solutions are diluted with distilled water to half strength The epithelium swells about 20 μ and both potential and current rise sharply and remain at fair levels for almost an hour Then both bathing solutions are made ordinary Ringer (with normal K concentration) The epithelium shrinks and both potential and current drop somewhat The current how ever seems to rise again toward the end of the experiment and at the same time there seems to be a slight increase in the epithelial thickness

It seems a fair conclusion that the depression of the potential and the short circuit current which is known to occur in K free solutions in part at least is due to the shrink age since it can be reversed by dilution In this context it may be mentioned that skins in K free sulphate Ringer do not shrink and although their active sodium transport is ultimately inhibited they stand up much better than the skins in K free chloride Ringer

In the experiment with gluconate replacing chloride shown in Fig 2 we apparently have another example of shrinkage due to loss of KCl Here however it is chloride which is lacking in the medium and thus reduces the activity of KCl to zero Whether the sharp drop in short circuit current resulting from the switch from chloride to gluco-nate can be attributed to shrinkage is uncertain It is however suggestive that when at the fourth vert cal l ne the half strength gluconate is replaced by full strength chlo-ride there is first a shrinkage probably due to loss of water follov ed by a swelling which must be due to uptake of chloride This latter swelling is accompanied by an increased current

COMPLEX SHRINKAGE AND SWELLING PATTERNS

a Chloride Ringer, made hypertonic with urea

As mentioned above there are indications that urea can penetrate to some extent into the epithelium when added from the outside When urea is added to the inside its ability to diffuse into the cell becomes even more apparent and gives rise to complex shrinkage and swelling patterns Fig 7 shows a typical experiment The current is recorded continuously whereas the potential is read at the times indicated with crosses At the first vertical line the inside solution is made double isotonic by addition of urea (220 mmoles per liter) The epithelium shrinks for about 10 min but then starts swelling again probably due to entry of urea in the cells It is seen that the short circuit current follows the changes in epithelial thickness quite well This experiment seems to indicate that it is the volume change rather than the hypertonicity *per se* which influences the short-circuit current The potential changes seem to parallel the changes in current At the second vertical line urea is also added to the outside medium There is a slight shrinkage but both potential and current increase somewhat and then remain constant We see here for the first time a deviation from the rule of a close correlation between current and epithelial thickness So far such deviations have only been seen in some but not all of the experiments with urea added to the inside The significance of this phenomenon will be discussed below At the third vertical line ordinary Ringer replaces the hypertonic solution on the inside The skin first swells and then shrinks just as one would have anticipated assuming that the cells would first take up water and then lose urea These changes are associated with similar changes in the short-circuit current

The potential reacts in a most spectacular way dropping first steeply from 102 mV to 45 mV and then levelling off at 22 mV Thus urea when present on the outside only depresses the potential much more than the current In other words the d c skin resistance is strongly reduced in agreement with previous observations

The volume reponses produced by addition of urea to the inside solution fall in two classes Most of the responses resemble that shown in Fig 4 Some skins after the initial shrinkage swell to a thickness definitely larger than the original one This phenomenon which might be called an anomalous swelling has been observed in 10 cases all in one batch of frogs The thickness at the maximum of swelling exceeded in these skins the original thickness before urea by 10 $\mu \pm 3 \mu$ The phenomenon was reproducibl in the skins which gave it. We shall return to this phenomenon in the discussion

b Chloride Ringer made hypertonic with KCl

According to MacRobbie and Ussing (1961) the inward facing side of the epithelium must be permeable to chloride as well as potassium because the epithelium swells when part of the Na in the Ringer is replaced by potassium in the inside solution Therefore KCl should behave rather like urea when it is added to the inside chloride Ringer to make it hypertonic This is usually what happens Fig 8 shows a typical experiment Originally both sides of the skins are bathed with chloride Ringer At the first vertical line the inside solution is made double isotonic by addition of 120 mmoles of KCl per liter The epithelium shrinks about 10 μ in 10 min and then slowly swells to its original volume The short-circuit current first is strongly depressed but as the volume increases again the current recovers partly When ordinary chloride Ringer replaces the hypertonic solution at the second vertical line the epithelium swells about 15 μ after 6 min

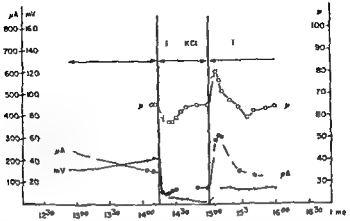

Fig 8 Effects of KCl added to the Ringer solution bathing the inside of the frog skin (Rana temporaria) upon short-circuit current (μA) skin potential (mV) and epithelial thickness (μ) Exposed area 7 cm Outside bathing solution = chloride Ringer throughout Inside bathing solution initially chloride Ringer At first vertical line 115 mmoles of KCl per liter are added to the inside solution At second vertical line the inside solution is again made ordinary chloride Ringer

and then declines again to reach the control value after about two hours What happens evidently is that the cells first swell because water can enter faster than KCl can leave the cells During this sequence of events the short-circuit current follows the volume changes increasing and decreasing as the epithelial thickness does so Also the potential undergoes variations which seem closely related to the epithelial volume

In this experiment it may not be justified to equalize short-circuit current and active sodium transport, because during the period of increased KCl-concentration on the inside the diffusion outward of chloride may have increased the current more than the diffusion of potassium has reduced it The close correlation between volume changes and changes in short-circuit current is however very spectacular One case of anomalous swelling was seen in five experiments of this type

Discussion

A comparison of the different volume responses presented above makes it apparent that they can be predicted at least qualitatively if we make the following two assumptions 1) Swelling increases the active sodium transport whereas shrinkage decreases it 2) Hypertonicity makes the outward facing boundary of the epithelium more leaky to water and passively diffusing ions

With respect to the first mentioned thesis it seems obvious that a close correlation exists between changes in epithelial volume and changes in the rate of active transport of sodium as measured by the short-circuit current

This finding has a bearing on many types of experiments with frog skin preparations For instance it has long been known that the frog skin potential is depressed in the absence of potassium in the inside medium (Fukuda 1942) and that the active sodium

transport depends on potassium in the inside medium (Huf and Wills 1951). It now appears (see Fig. 6) that the lack of potassium inhibits sodium transport, at least in part by way of the shrinkage which results from loss of KCl from the epithelial cells. This conclusion is strongly supported by the fact that the sodium transport returns when the epithelium is induced to swell by dilution of the K-free Ringer. It is significant that if K-free sulphate Ringer is used, there is no shrinkage for many hours and although the sodium transport is ultimately inhibited, it takes many hours of K-deficiency.

At first sight the finding that the sodium transport can go on for hours in K-free sodium sulphate or dilute NaCl Ringer might seem to be definite proof against the Na/K exchange hypothesis (Koefoed-Johnsen and Ussing 1958). Such an argument would be valid if the inside bathing solution were in direct contact with the transporting cells. This is probably not the case, however. Much evidence has accumulated (Ussing 1964, Ussing and Windhager 1964, Farquhar and Palade 1964) that it is the outermost living layer of cells in the epithelium which forms the sodium selective, potassium impermeable barrier in the skin, and that sodium can diffuse from one cell to the next by way of intercellular bridges. According to Farquhar and Palade (1964) and Wingstrand (personal communication) all the ATPase of the epithelium is located at the cell membrane facing the interspaces of the epithelium and not the basement membrane. This might indicate that the active sodium transport pumps sodium into the interspaces which communicate through openings between the basal cell layer with the inside bathing solution. If the potassium exchange with the cells is also located in the interspaces, this ion might therefore have a measurable concentration at the level of the "pump" even when the inside bathing solution is K-free. Thus it remains an open question whether or not the sodium pump of the frog skin is a Na—K exchange pump.

While the epithelium shrinks when the inside K-concentration is lowered, it swells when inside Na is replaced more or less completely with K. It has been shown by ...cker et al. (1963) that with NaCl Ringer outside and KCl Ringer inside there is short-circuit current of almost normal magnitude due largely to active transport of sodium inward. This current increases with time. The same authors (Ussing et al., in preparation) later showed that if the swelling is inhibited by using sulphate instead of chloride in the bathing solutions, the short-circuit current does not increase with time. If, however, antidiuretic hormone is added to the potassium sulphate Ringer on the inside, the epithelium starts swelling, and the short-circuit current increases. Thus the relationship between short-circuit current and epithelial volume manifests itself even in this peculiar system.

The experiments with hypertonicity produced by glucose on the inside and with dilution of the inside solution call for little comment. The correlation between volume changes and changes in short-circuit current are very apparent and it is also seen that the correlation between volume and potential is much less apparent. Indeed in the experiments with dilution of the inside solution the correlation between volume and potential is very poor while the correlation volume-current is excellent. In this study the dilution has not been carried beyond that of "half sulphate Ringer". The skins often suffer irreversible damage at higher dilutions. With respect to the effect of hypertonicity on the outside, there are characteristic differences between the effects of the osmotically active agents used. Thus the shrinkage seems more pronounced when the hyperosmolarity is produced by sugars or NaCl, less pronounced when produced by more diffusible substances like urea and glycerol and totally absent with ethyl alcohol. Similarly, the inhibition of active sodium transport is only seen in the cases of the less

diffusible substances Indeed with urea there is usually no inhibition whatsoever (Ussing and Windhager 1964) Thus one gets the impression that the degree of shrinkage is determined by the effective osmotic pressure and that the shrinkage in turn determines the reduction of the short-circuit current It should be mentioned that in rare cases of the hypertonicity on the outside produced by urea there is an increase in short-circuit current despite a slight shrinkage (see Fig 7) This might indicate a stimulating effect of urea on the active sodium transport which is normally compensated by the inhibition due to shrinkage However the phenomenon may have a different explanation

The setting of the rate of the sodium pumping by the cell volume would under most conditions give a feed back regulation of the water and electrolyte content of the cell The physico-chemical reasons for the relationship remain to be clarified Shrinkage of the cell membrane might close the pores whereas pores may be opened by stretching [1] Furthermore the viscosity of the cytoplasm probably depends on the water content of the cell and would determine not only the rate of diffusion of sodium but also that of ATP or other cell constituents necessary for the active transport

Clearly the fact that the active sodium transport usually varies in concordance with the degree of swelling of the whole epithelium does not necessarily mean that it is the total thickness of the epithelium which determines the rate of transport It stands to reason that all epithelial cells have similar osmotic properties so that as long as they are exposed to similar conditions they react osmotically in about the same way Now as already mentioned there are reasons to believe that all layers of epithelium cells in the frog are in diffusion contact with the inside bathing solution via the interspaces and the relatively permeable basement membrane Therefore it could very well be that the control of entry of sodium into the epithelium from outside depends on the degree of swelling of the outermost living cell layer but that its volume happens to vary roughly in proportion to that of the entire epithelium when the osmotic conditions are changed This interpretation gains support from the observation (Hoshiko 1961) that the main resistance of the skin is located towards the outside Also the few cases of apparent lack of correlation between volume change and change in active sodium transport may find a natural explanation if we assume that the active transport is controlled by the outermost cell layer This may become more apparent when we have discussed the second one of the above mentioned theses that hypertonicity makes the outward facing boundary of the epithelium more leaky to water and passively diffusing ions

That the water permeability is increased by hypertonicity is evident from the fact that there is no measurable swelling when the outside medium is made hypotonic whereas hypertonicity of the outside medium does give rise to shrinkage This again means that whereas the inner boundary of the epithelium is normally more permeable to water than the outer one (MacRobbie and Ussing 1961) the opposite is the case when the outside medium is hypertonic Such an effect of hypertonicity would also explain the phenomenon of anomalous swelling which is sometimes seen when the inside solution is made hypertonic with urea or KCl If hypertonicity makes the outer epithelial border more permeable to water than the inner border a swelling will ensue if two conditions are fulfilled Firstly the substance making the inside solution hypertonic should be able to penetrate into the cells and secondly the substance should

[1] This would imply that swelling and shrinking also should affect the cellular permeability to water and passive ions The epithelial permeability for these substances is however likely to possess both cellular and intercellular components In several of the conditions studied here the latter one seems to dominate the picture

not leak out too readily through the outer boundary Anomalous swelling should on the other hand never occur if the *hypertonicity is produced by* substances which can not penetrate into the cells Anomalous swelling has indeed never been observed in a large number of experiments with glucose and sucrose *but only with the relatively* diffusible substances urea and KCl Hypertonicity on the outside as well as hyper tonicity produced by urea and KCl on the inside thus increase *the water permeability* of the outer boundary of the skin

Hypertonicity especially of the outside solution also increases the passive leakage of ions through the skin With hypertonicity outside produced by urea (sulphate Ringer on both sides) the passive leakage of sodium outward as well as the leakage of sulphate inward are enormously increased (Ussing and Windhager 1964) This leakage represents a shunt for the sodium transport battery and its effect is reflected in the violent drop in potential in spite of a virtually unaltered rate of active sodium transport Other osmotically active substances like NaCl and sugars also increase the shunt when added to the outside solution as evidenced by the fact that they lower the potential relatively more than the short circuit current (see Fig 4 and 5) This fact makes it less attractive to try to explain the increased shunt produced by hypertonicity in terms of a specific chemical action In this context it may also be pertinent that if urea is first added to the inside solution of the skin and allowed to equilibrate addition of urea to the outside does not give rise to the customary breakdown of the potential despite the *fact that the urea concentration at the outer boundary must have been as high at least* at the outer boundary as when urea is added at the outside only As an alternative to *the assumption of a chemical effect on the outside of the skin of the osmotically active* substances one is therefore tempted to look for an osmotic mechanism It is for in stance possible that osmotic shrinkage of the outermost layer of cells might put a strain the seal between them so that it becomes leaky Hypotonicity of the outside solution ld on the other hand make the outermost cells swell slightly which should make he seal tighter According to Farquhar and Palade (1964) the interspaces are normally closed towards the outside by close attachment of the cells of the outmost layer to each other If the permeability of this seal can be increased when it is put under mechanical strain we would have a simple explanation of the variations in water permeability and electrical shunt It may be pertient in the context that according to Motokawa (1933) the potential across a skin with ordinary Ringer on both sides will drop reversibly when the skin is made to bulge by applying a hydrostatic pressure head on either inside or outside Such a treatment would also tend to strain the intercellular seals

If the cells of the outermost layer exhibit such a valve effect closing the *inter* spaces toward the outside as long as the outside medium is hypotonic towards the cells but opening a path *from the outside to the interspaces when the outside becomes* hypertonic the permeability changes to water and passively diffusing ions would find a simple explanation *Electron microscopic* studies are being planned to find out whether the predicted volume changes of the outermost cell layer do indeed take place

As mentioned above some of the rare cases of lacking correlation between degree of swelling and short-circuit current may find a natural explanation if we assume that it is *the osmotic reaction of the outermost cell layer which determines the short-circuit* current If we consider for instance the response to addition *of urea on* the outside in *Fig 7 at the second* vertical line there is a slight shrinkage but the current increases However the relative permeabilities of the outermost cells to urea and water at *their* outer and inner boundaries might well be such that they *attain* a larger volume when

exposed to urea on both sides than when exposed at the inside only. However, for the time being it cannot be excluded that urea besides its general osmotic action does increase the permeability of the outer surface of the epithelium to sodium. If so, the effect would normally be just about balanced by the inhibition of sodium transport produced by the slight osmotic shrinkage which this solution produces.

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The Utilization of a Presumably Low-Cariogenic Carbohydrate Derivative

By

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Abstract

Dahlqvist A and U Telenius *The utilization of a presumably low-cariogenic carbohydrate derivative* Acta physiol scand 1965 63 156–163 — The digestion and absorption of a presumably low cariogenic carbohydrate derivative a hydrogenated partial hydrolyzate of starch has been studied This contains free sorbitol plus disaccharides oligosaccharides and dextrins in which [illegible]se molecule which had a free aldehyd group prior to hydrogenation has been reduced [illegible]ol The dextrins present are rapidly hydrolyzed by α amylase under the formation [illegible] and oligosaccharides Those disaccharides which are composed only of glucose are com[ple]tely hydrolyzed by the intestinal disaccharidases and rapidly utilized in the body The [di]saccharides containing one molecule of glucose and one of sorbitol either initially present as disaccharide or formed on the enzymatic cleavage of the dextrins and oligosaccharides are very slowly hydrolyzed The glucose bound in this way largely escapes absorption The sorbitol whether free or bound is very slowly absorbed

A new kind of carbohydrate material for the fabrication of sweets has recently appeared which is prepared by the hydrogenation of partially hydrolyzed starch This substance has been found to cause little or no pH fall in the dental plaque material on oral application and is therefore believed to be low-cariogenic (Frostell 1963) In the present investigation the utilization of this modified carbohydrate material has been studied in rats Some *in vitro* incubations have also been performed with human enzyme preparations in order to obtain information about the digestibility of the hydrogenated starch hydrolyzate in man

Materials and methods

Carbohydrates

Substance S is the new carbohydrate material obtained by the hydrogenation of a starch hydrolyzate It contains sorbitol plus a mixture of di and oligosaccharides and dextrins in which that glucose component which previously had a free aldehyde group has been reduced to sor

b tol *Substance G* is the starch hydrolyzate from which substance S is prepared Substance G contains glu ose di and ol gosaccharides and dextrins Substance S and substance G were obtained in the form of spray-dried powders from Lyckeby Starkelseforadling AB Lyckeby, Sweden

Disaccharide S is the disaccharide fraction from substance S *Dextrin S* is the oligosaccharide dextrin fraction (containing tri and higher oligosaccharides plus dextrins) from substance S *Dextrin G* is the (tri and higher) oligosaccharide-dextrin fractio i from substance G These were isolated by carbon-celite col imn chromatography (Whistler and Durso 19;0) and characterized by paper chromatography (see below)

Starch (amylum solubile siccum nach Zulkowsky) was obtained from Merck AG (Darmstadt Germany) *glucose* from B ker Chemical Co (Phill psburg N J., U S A) *sorbitol* and *maltose* from Pfanstiehl Chemical Co (Waukegan Ill U S A.)

Animals

Alb no rats of the Sprague Dawley strain were used They were fed a commercial pellet diet Before the experim ents the animals were fasted for 20 hours w th free allowance for water

Analytical methods

1) *Free glucose* was assayed with the tris-glucose oxidase reagent (Dahlqvist 1961) Sorbitol does not react with this reagent

2) *To l glucose* (free pl us bound glucose) was assayed with either one of the follow ng two methods

a) With the tris-glucose oxidase reagent after that the preparation had been boiled with 1 N HCl for 60 m n and then neutralized with NaOH

b) Wi h the anthrone reaction performed as described by Scott and Melvin (19;3) but with heating at 100 C for 7.5 m n Sorb tol d es not react with the anthrone reagent

3) *Bound glucose* was calculated as the difference bet veen total glucose and free glucose

4) *Sorbitol* was assayed with the m ethod of Corcoran and Page (194;) after that the preparation had been hydrolyzed with acid as for the assay of total glucose described above Glucose will also react with the periodate — chromotropic acid reagents used for the assay of sorbitol but the reaction of glucose is slow and the extinction coefficient obtained for glucose was only o of that for sorbitol which implies that sorbitol can be assayed specif cally in the presence of up to 2—3 tim es the same amount of glucose Free and bound so rb tol were not assayed separately

5) *Reduc g power* was assayed with the 3 5-d n trosalicylate reagent (Sumner 1924 Hostettler Borel and Deu l 1951) Standard curves were prepared both with glucose and maltose.

6) *Paper chromatography of sugars* Descendent chromatograms were run on Whatman No 1 filter paper using n butanol ether water (40 10 50 upper phase) as the solvent The dr ed chromatograms were sprayed w th a m ixture of equal parts of 0 1 N $AgNO_3$ and 5 N NH_4OH and heated for 5—10 min (Partridge 1948) Sorbitol and sorbitol-containing ol gosaccharides react equally well s reduc ng sugars

7) *Protein* was assayed with the method of Lowry *et al* (1951 with the mod ficat on introduced by Eggstein and Kreutz (1955) A standard curve was prepared with freshly dissolved human serum albumin (AB Kab Stockholm Sweden)

Digestion studies in vitro

Homogenates of rat small intestinal mucosa rat pancreatic tissue and human small intestinal mucosa (obtained from a piece of surgically removed human lower duodenum and upper jejunum) were prepared with an Ultra Turrax homog nizer The tube was chilled with ice during hom genization Human saliva was collected after rinsing the mouth with water The preparations were diluted with water or 0.9 NaCl to give an appropriate enzyme concentration and then incubated with the different carbohydrates studied Incubation was performed at 37 C in either 0 05 M sodium maleate buffer pH 6 5 or 0 025 M sod um potassium phosphate buffer pH 6 9 When starch substance S substance G dextrin S or dextrin G was used as the substrate sufficient sodium chloride was always added to ensure the activation of α amylase (Meyer Fischer

and Bernfeld 1947) When incubation was performed for more than 1 hour toluene was added as a preservative The increase in reducing power was assayed with the 3 5-dinitrosalicylate and the amount of glucose liberated with the tris glucose oxidase reagent

Digestion and absorption studies in vivo

1) *Intestinal digestion and absorption* The same method as has earlier been used to study the digestion and absorption of sucrose maltose and trehalose in the rat utilizing polyethylene glycol as an unabsorbable reference substance (Dahlqvist and Thomson 1963 a—b) was used to study the digestion and absorption of substance S and substance G The rats weighed about 200 g and each animal was fed 0 800 gram of the substance to be tested The assay of sorbitol and free and bound glucose in the samples obtained was performed with the methods described above *Protein precipitation was not performed* since this in preliminary experiments was found to remove part of the dextrins

2) *Deposition of liver glycogen* *Fasting rats weighing 250—300 g were lightly* anesthetized with ether and fed 1 50 g of either substance S or substance G in 5 ml of water by a stomach tube Control animals were fed the same volume of 0 9 NaCl After 4 hours with free access to tap water the animals were killed by a blow on the head The liver was immediately removed and cut into small pieces in a tube with 30 KOH The tube was weighed before and after the addition of the liver and the wet weight of the liver was calculated The tubes were then heated in a boiling water bath for 2 hours cooled and their contents diluted to 100 ml with water The glycogen was then assayed in the following way an aliquot of the solution containing 50—150 μg glycogen was diluted with water to 1 0 ml and 1 6 ml of 95 ethanol was added The tubes were left at room temperature for 3 hours Then they were centrifuged at 1 000 × g for 10 min and decanted The sediment was suspended in 2 5 ml 60 ethanol and the tubes again centrifuged and decanted The precipitate was dried at C suspended in 2 0 ml of water and the glycogen assayed with the anthrone reagent A standard curve was prepared with glucose 90 μg of glycogen gives the same reading as 100 μg of glucose

Results

Composition of substance S and substance G

Substance S contained no detectable free glucose 64 2 % bound glucose and 28 9 sorbitol (free plus bound) It has a very weak reducing power 1 mg had the same reducing power as 0 007 mg maltose

Substance G contained 7 1 free glucose and 91 1 bound glucose It has a relatively strong reducing power 1 mg had the same reducing power as 0 7 mg maltose

On paper chromatography both substance G and substance S showed one monosaccharide spot (glucose and sorbitol respectively) one disaccharide spot and a series of oligosaccharide-dextrin spots

Digestion studies in vitro

1) *Digestion by a mucosal homogenate of rat small intestine*

Fig 1 shows the course of the hydrolysis *in vitro* of substance S by a mucosal homogenate from rat small intestine In the beginning of the reaction the increase in reducing power per time unit was much larger than what corresponded to the amount of glucose

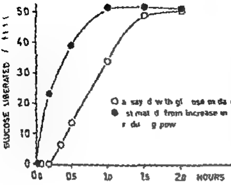

Fig 1 The hydrolysis of substance S by a homogenate of rat small intestinal mucosa The reaction mixture contained 10 mg/ml of substance S and 1 mg/ml of mucosal protein in 0.05 M sodium maleate buffer pH 6.5 It also contained 0.4 mg/ml of NaCl which activates the α amylase Incubation was performed at 37 C The increase in reducing power was measured with the 3 5-dinitrosalicylate reagent and the glucose liberated with the tris glucose oxidase reagent

liberated Presumably this is caused by the action of α amylase which hydrolyzes the dextrins to reducing di and oligosaccharides Then the oligosaccharides were hydrolyzed to glucose by the intestinal disaccharidases and the curves for increase in reducing power and free glucose approached each other After 2 hours 50 % of the glucose had been liberated On prolonged incubation the reaction slowly proceeded further but still after [illegible] hours only 80 % of the total glucose had been liberated both as calculated from the reducing power and from the enzymatic assay of free glucose

On incubation with substance G under the same conditions the velocity and pattern of the initial phase of the reaction were the same as for substance S but in 20 hours the hydrolysis of substance G to free glucose proceeded to 100 %

Incubation with the isolated dextrin S and dextrin G respectively gave the same results as for each of the corresponding substances above

Incubation with the isolated disaccharide S revealed a very slow hydrolysis of this substrate During the hydrolysis of disaccharide S the rate of glucose liberation was only 1/60 of that from maltose incubated under the same conditions Since disaccharide S contains only one molecule of glucose and maltose contains two this means that in terms of the number of glycosidic linkages split the hydrolysis of substance S proceeds with a rate which is 1/30 of that for maltose On long incubation with concentrated enzyme preparations the hydrolysis of disaccharide S proceeded to completion

It was also investigated whether disaccharide S inhibits the maltase activity of the mucosal homogenate In this experiment the reaction mixture contained either 10 mg ml of maltose or 10 mg/ml each of maltose and disaccharide S 0.04 mg ml of mucosal protein and 0.05 M sodium maleate buffer pH [illegible] 5 Incubation was performed at 37 C. The amount of glucose liberated when only maltose was present was 126 μg/ml reaction mixture/hour and when maltose and disaccharide S were present in mixture 108 μg Thus under these conditions disaccharide S only inhibited the maltase activity by about 14 %

?) Digestion by a mucosal homogenate from human small intestine

A homogenate of human small intestinal mucosa hydrolyzed substance S in the corresponding way as described for the rat mucosal preparation above The hydrolysis of disaccharide S by the human preparation proceeded with a rate which was 1/40 of that for maltose

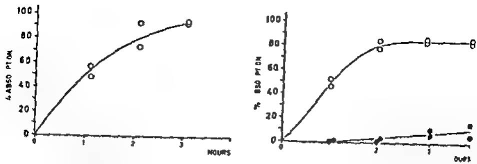

Fig 2 Total absorption of 0 800 g of substance G (left) and substance S (right) in 200 g rats After the ingestion of substance S the absorption of the glucose (○) and sorbitol (●) components have been studied separately

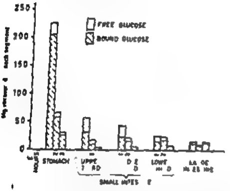

Fig 3

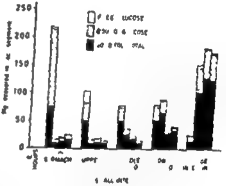

Fig 4

Fig 3 Mean amounts of glucose (free and bound) recovered from the different parts of the gastrointestinal tract after the feeding of 0 800 g of substance G to 200 g rats

Fig 4 Mean amounts of glucose (free and bound) and sorbitol (total) recovered from the different parts of the gastrointestinal tract after the feeding of 0 800 g of substance S to 200 g rats

3) *Digestion by a homogenate of rat pancreas*

The reaction mixture contained 10 mg/ml of substance S substance G or starch 0 003 mg/ml of tissue protein 0 02 mg/ml of NaCl and 0 025 M sodium potassium phosphate buffer Incubation was performed at 37 C The increase in reducing power when substance S was used as the substrate was about 1 3 of that when starch was used The hydrolysis of substance G proceeded at about the same rate as that of substance S and thus the slower hydrolysis of these substances as compared with starch seems to be caused by their smaller molecule size Paper chromatography showed disaccharides to be the main hydrolysis product

Maltose was hydrolyzed by the pancreatic homogenate with a rate which was only 1/3 000 of the rate of hydrolysis of starch expressed as the number of glycosidic linkages split per time unit Disaccharide S was not hydrolyzed at a measurable rate (less than 1/100 of the rate of hydrolysis of maltose)

TABLE I Deposit on of liver glycogen after the feed ng of 1 50 g of substance G or substance S to fasting rats

Substance fed	Wet v eight of liver (g)	Total amount of glycogen found (mg)	Glycogen as % of the wet weight of the li er	
NaCl (controls)	9 59	25	0 26	0 32
	10 11	27	0 27	
	9 73	32	0 33	
	9 61	39	0 41	
Substance G	9 87	290	2 95	3 47
	10 67	250	2 35	
	9 47	367	3 88	
	11 34	530	4 69	
Substance [redacted]	9 83	359	3 65	3 74
	8 75	364	4 10	
	11 67	367	3 15	
	11 23	453	4 04	

4) *Digestion by human saliva*

The reaction mixture for studying the hydrolysis of substance S substance G and starch contained 0 007 mg/ml of protein The incubation conditions were the same as described for the rat pancreatic homogenate above Like the rat pancreatic homogenate the human saliva hydrolyzed substance S and substance G with a rate which was about 1/3 of the rate of hydrolysis of starch Paper chromatography showed that disaccharides were the main hydrolysis product

The saliva had very weak maltase activity 1/10 000 of its amylase activity and no detectable activity towards disaccharide S (less than 1/50 of the maltase activity)

Digestion and absorption in vivo

1) *Intestinal digestion and absorption*

Fig 2 shows the total absorption of the [redacted] 800 g dose of substance S and substance G when fed to 200 g rats The absorption of substance G proceeded rapidly and the curve was similar to that earlier obtained for glucose and maltose (Dahlqvist and Thomson 1963 b) The absorption of the glucose and sorbitol components of substance S were studied separately The glucose component was absorbed rapidly until about 85 of the total glucose had been absorbed Then practically no further absorption of glucose occurred The sorbitol component was absorbed very slowly After 4 hours still only a few of the sorbitol had been absorbed

Fig 3 and 4 show the amount and kind of carbohydrate recovered from the different parts of the gastrointestinal tract after the feeding of substance G and S respectively After the ingestion of 0 800 [redacted] of substance G very little sugar was recovered from the

distal part of the intestine but after 0.800 g of substance S large amounts of sorbitol and some glucose reached the lower part of the small intestine and the large intestine. Essentially all of the glucose that reached the large intestine was bound glucose and there seems to be reason to assume that this glucose represents the fraction which is bound directly to sorbitol since the *in vitro* experiments showed that this link is split very slowly.

The % absorption of the carbohydrate in the samples obtained from the different parts of the intestine calculated from the amount of polyethylene glycol recovered agreed very well with the data above. After the ingestion of substance G over 90 % had been absorbed in the samples which reached the lower part of the small intestine and the large intestine. After the ingestion of substance S 75—80 % of the glucose and only a few % of the sorbitol had been absorbed in these samples.

No difference could be seen between the rats fed substance S and those fed substance G in the rate of stomach emptying calculated from the polyethylene glycol. The passage from the small intestine into the large intestine was somewhat more rapid when substance S was used.

2) *Deposition of liver glycogen*

Table I shows the amounts of liver glycogen deposited 4 hours after the ingestion of 1.50 g of substance S and substance G respectively to fasting rats. The increase in liver glycogen content was equally large after both substances.

Discussion

Both the *in vitro* and *in vivo* experiments show that 80 % of the glucose in substance S (which equals half of its total weight) can be easily split off by the digestive enzymes and absorbed and utilized in the body. The hydrolysis occurs in two steps: first the dextrins are split into di- and oligosaccharides by the α-amylase and then those of the di- and olisaccharides which are composed only of glucose are hydrolyzed to glucose by the intestinal disaccharidases.

The disaccharides composed of one glucose and one sorbitol molecule in contrast are split very slowly and the glucose bound in this way largely escapes being absorbed in the small intestine. Whether tri- and higher oligosaccharides containing sorbitol also are split more slowly than the corresponding sugars built up of only glucose cannot be stated at present since these have not been isolated.

The sorbitol, whether free or bound, is absorbed very slowly.

The fact that substance S is split by α-amylase in the saliva under the formation of maltose and related oligosaccharides and nevertheless does not decrease the pH of the dental plaque material opens the question whether the maltase activity or the glycolytic reaction sequence in the dental bacteria are inhibited by sorbitol or some of the sorbitol-containing saccharides. This point calls for further investigation. The intestinal mucosal maltase activity was found to be only slightly inhibited by the glucose-sorbitol disaccharides.

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Acta physiol scand 1965 63 164—170

From the Institute of Zoophysiology University of Oslo Blindern Norway

Comparative Aspects of the Respiratory Gas Exchange of Sea Urchins

By

JOHAN B STEEN

Received 1 June 1964

Abstract

Steen J B Comparative aspects of the respiratory gas exchange in the sea urchin Acta physiol scand 1965 *63* 164—170 — Respiration of the sea urchin *Strongylocentrotus droebachiensis* has been studied with measurements of oxygen uptake at different ambient temperatures and with parts of the surface organs selectively covered with wax It was confirmed that the tube feet serve as the main respiratory organ At 16 C the oxygen uptake is reduced if a small pr j rt n the tube feet surface area is covered or if the external oxygen tension is reduced At C a larger decrease of podial area or tension is needed to reduce the oxygen uptake [illegible] lated oxygen uptake based on podial dimensions and measured oxygen tension differences is about ten times larger than the measured oxygen uptake The inability of the sea urchin to utilize its theoretical respiratory capacity is tentatively explained as a consequence of its poorly developed circulatory system with the lack of a respiratory pigment Comparison of the respiratory data from sea urchins with similar data from the eel reveal some trends in the evolution of respiratory mechanisms

During the evolution of aquatic respiration highly efficient conditions for gas exchange have been achieved in the teleostean gill *Here water and blood exchange gases as they* pass counter-current to each other at the respiratory membrane (the lamellae The presence of hemoglobin greatly *increases the oxygen capacity in the blood* It also influences the oxygen tension difference across the gill membrane in a favourable way in that relatively *much oxygen may be taken up at a small increase in the blood* oxygen tension

In comparison *the respiratory system of Echinoderms* is far *more primitive* In the starfish and the sea urchin the podia (tube feet) consitute the only respiratory surface of significance (Koller and Meyer 1933 Farmanfamaian 1958 For these animals the external medium is renewed only by movements of the podia while the internal circulation is very *primitive* (Fig 1)

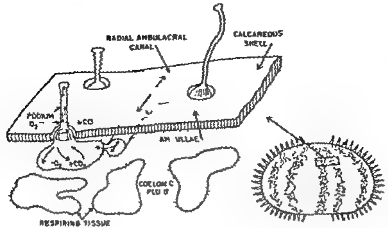

Fig 1 The c rculatory system of the sea urchin with section through one podium and its ampullae connected to the radial water canal

The circulatory fluid of sea urchins does not contain any respiratory pigment. In contrast to eel blood which when saturated with air at a normal pH may contain 14 vol % oxygen (Steen 1963 a) the ambulacral fluid will hold no more oxygen than the 0 06 vol % which is contained in salt water saturated with air or more than 20 times less than the blood of the eel

In the present investigation an attempt has been made to obtain quantitative data on the respiratory capacity of sea urchins These data have been compared with similar data on the eel in order to elucidate the different types of respiratory systems found in these animals and possibly contribute to our understanding of the evolution of re spiratory systems in general

Material

Sea urch ns *St o gylocent tus d oebak ensis* were obtained n the Oslofjord during February and March They were kept in natural sea wate cooled to 4—6 C

Methods

Oxygen uptake was measured by the mod fied Scholander espirometer (Steen and Iversen 1964)

The oxyg n content of the ambulacral flu d was measured according to the techn que of Schol nder et al (1953)

Melted Histowax was u ed to cover lected parts of the external surface of the an mals. In o der to edu e ill effects of the heat as l ttl wax a poss ble was applied at a time This some t mes resulted in incomplete covering

Podia and gills were fixed in situ by mmers on of th an mal in Bou n fluid To obta n extended podia the animal was f rst anesth tized n a solut on of l urethane in sea water whereupon the an mal was transferred to Bou ns flu d The a ea of the pod surface was estimated by count ng the podia and measuring unde m croscope the external dimensions in l ving specim n

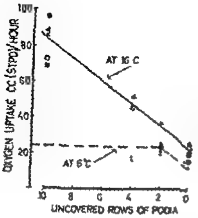

Fig 2 The effect of covering varying proportions of the tube feet surface of sea urchins at 6 C and at 16 C. The oxygen uptake of animals with all podia covered is due to incomplete contact between the podia and the wax

The structure of the respiratory and circulatory system

The sea urchin is covered by a calcareous shell a test with polar openings for mouth and anus Around the mouth small fingerlike structures protrude These are called gills although their respiratory function is doubtful The podia or feet are found in 5 double meridian rows Each podium penetrates the test by a two-hole pore (Fig 1) Inside the test the podium ends in an ampullae The ampullae from neighbouring podia lie tightly side by side and connect to a common radial canal The five radial canals connect to a circular canal around the mouth The fluid inside this system (ambulacral fluid) is circulated by ciliary movements Simultaneous movements of fluid in both directions is easily observed inside a podium It is thought that fluid passes from the ampullae into the podium through the one hole of the pore and back again through the other The circulatory system of the sea urchin is thus composed of several microcirculation units each consisting of a podium and an ampullae There is no capillary system Gas exchange probably occurs directly between the ampullae and the coelomic fluid which fills the internal spaces of the animal not occupied by other organs The coelomic surfaces are all ciliated Neither the circulatory fluid nor the coelomic fluid contain respiratory pigments (see Hyman 1955 pp 468—473)

Experimental results

Experiment I

In the first series of experiments the oxygen uptake of sea urchins at different temperatures was related to the area of their respiratory membrane The area was varied by covering increasing proportions of the podia by wax

The experiment started by continuous measurements of the oxygen uptake of intact animals until the uptake became stable usually after 1½ hrs Four six or eight rows of podia were then covered and the oxygen uptake again recorded Finally the remaining podia were covered step-wise and the oxygen uptake recorded after each treatment The experiment was performed at 16 and 6 C No reduction in oxygen uptake could be demonstrated following waxing of inter podial areas or the gills Covering the entire animal reduced the oxygen uptake to zero Fig 2 shows the influence of covering the podia

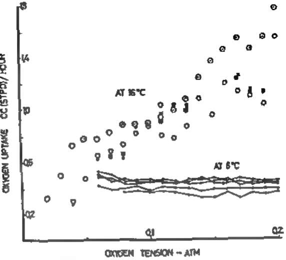

Fig 3 The effect of reduced external oxygen tension on oxygen uptake of intact sea urchins at 6° C and at 16° C

The effect of covering increasing proportions of the podia clearly depends upon the temperature and thereby upon the oxygen demand of the animal At 16° C the oxygen uptake is proportional to the uncovered proportion of podia At 6° C however 4/5 of the podia can be covered without any measurable effect on the oxygen uptake Only when the fifth and last pair of podia rows are covered does the oxygen uptake decrease

When the wax was removed the oxygen uptake would in most cases increase although never to the value recorded before any wax was applied

The results indicate that the respiratory membrane area is a limiting factor for oxygen uptake in the sea urchins only at a fairly high oxygen demand At 6° C the podial surface is apparently 4 times larger than required to cover the oxygen demand One might expect therefore that at the high temperature the animal had mobilized all the mechanisms whereby it obtains maximum respiratory capacity If this is correct a reduction in the oxygen tension difference between internal and external medium should reduce the oxygen uptake at 16° C but not at 6° C

Experiment 2

The sea urchins were placed in respirometers filled with aerated water The syringe which normally contains oxygen was filled with nitrogen so that oxygen consumed by the animal was replaced by nitrogen The oxygen tension was thus reduced as the experiment proceeded The oxygen uptake was measured continuoulsy until 4/5 of the available oxygen had been used This type of experiments were performed at 6 and 16° C The results are shown in Fig 3 At 16° C the oxygen uptake is directly proportional to the external oxygen tension while at 6° C a reduction of external oxygen tensions down to 4/5 of the air value did hardly influence the uptake at all

At the end of the experiments the podia did not move as much as usual However when the animals were transferred to aerated water they soon regained their normal vitality

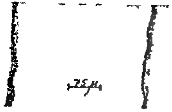

Fig. 4. A longitudinal section through a sea urchin tube foot.

Determination of respiratory capacity

The foregoing experiments indicate that at a temperature of 16° C the sea urchins utilize their functional respiratory capacity completely. For further analysis of this capacity a calculation of the oxygen uptake was attempted. The parameters required for this calculation, i.e. the area and thickness of the respiratory surface and the difference in oxygen tension existing across it, were determined.

Numerous estimations of the thickness of the podia wall gave an average of 15 μ. Fig. 4 shows part of a longitudinal section of a podium. A 70 g sea urchin has about 1 000 podia. When fully extended they may be 20 mm long and have a diameter of 0.4 mm. This gives an area of $3.14 \times 0.4 \times 20 \times 1\,000 \approx 25\,000$ mm² = 250 cm².

To determine the difference in oxygen tension acting across the podia membrane one should ideally know the oxygen tension of the ambulacral fluid as it enters and as it leaves the podia. Unfortunately such measurement could not be carried out with our methods. Instead we measured the oxygen tension of the coelomic fluid. This oxygen tension will certainly be lower than that of the fluid entering the podia from the ampullae since the coelomic fluid fills the space between the ambulacral system and the respiring tissues (Fig. 1).

The oxygen tension was determined indirectly. First the relation between oxygen tension and oxygen content of the coelomic fluid was established by analysis of fluid samples with known tension. This fluid was found to have the same oxygen solubility as water of the same salinity and temperature. Samples of coelomic fluid were then taken anaerobically from animals living in well aerated water at 6° C and 16° C and their oxygen content measured and the oxygen tension obtained. For three animals at 6° C oxygen tension varied between 0.02 and 0.015 atm, while for three animals at 16° C tensions were between 0.03 and 0.04 atm.

If we assume that the ambient oxygen tension is 0.21 atm and that the ambulacral fluid has an oxygen tension of 0.04 atm as it enters the podia and 0.20 as it leaves, the average oxygen tension acting across the podia membrane will be $0.21 - \frac{0.20 - 0.04}{2} \approx 0.13$. If we use the diffusion constant for muscle tissue of 0.14 ml O_2/min/cm²/atm/μ (Krogh 1941 p. 13) in the usual diffusion formula we get

$$\text{Vol } O_2 = 0.14 \cdot \frac{250 \cdot 0.13 \cdot 60}{15} \approx 19 \text{ ml } O_2/\text{hr}$$

In comparison the highest oxygen uptake recorded in this investigation was [illegible] ml O_2/hr for a 70 g animal at 13° C.

This discrepancy between calculated and measured respiratory capacity may be reasonably explained by the following factors although their relative importance is difficult to evaluate.

TABLE I

	Respiratory membrane		Oxygen gradient (atm)	Respiratory capacity (ml O_2/cm)	
	Area (cm)	Thickness (μ)		Calculated	Measured
Eel gills	530	7.5	0.13	0.15	0.15
Sea urchin podia	250	15	0.13	0.08	0.068
Sea urchin ampullae	65	15	0.04	0.015	0.03

First, in the calculation we assume all the podia to be fully extended. Direct observations show that this is not the case. It is, however, very hard to find an average degree of extension. Secondly, we assume the thickness of the podia walls in histological sections to be identical to the actual diffusion path. The tissue had, however, probably undergone some shrinking during fixation and imbedding. Furthermore, circulation of ambulacral fluid and renewal of external medium may be insufficient, whereby the actual diffusion path becomes larger than the anatomical. The lack of separated circulation paths from podia to tissue and back again may also act to render the actual oxygen tension gradient smaller than assumed in the calculation. There may thus be mixing and gas exchange between the fluid streams moving counter current to each other in the same podium.

One can also estimate the oxygen diffusion from the ampullae into the coelomic fluid. Dissections show that about one third of the internal surface of the sea urchin is covered by the podial ampullae. In a 70 g animal with a radius of 4 cm this corresponds to an area of about 65 cm. The average oxygen tension in the ampullae is maximally $\frac{0.20 - 0.04}{2} = 0.08$ *atm, which gives a tension difference with the coelom of* 0.04 atm. Inserting these data in the formula and still assuming a diffusion distance of 15 μ we get an oxygen diffusion of about 1 ml/hr.

Discussion

The podia of sea urchins are locomotive and sensory organs besides having their respiratory function. Such multiple function of one organ gives a primitive condition when compared with the situation in more specialized organs as gills. The organ is only partially adapted to each of its functions. Thus the wall of the podia is about 15 μ thick as compared to 7 μ for the respiratory epithelium of the eel gill (Steen and Kruysse 1964). This thickness may be necessary since there are both nerves and contractile elements in the podial wall.

In Table I data from the present investigation are compared to similar data obtained in the eel (Steen and Kruysse 1964). It can be seen that there is a major difference between the two respiratory organs, the primitive podia and the specialized gills: oxygen uptake in the eel may actually reach values corresponding to the theoretical respiratory

capacity of the gills whereas maximum oxygen uptake in the sea urchin is only about one tenth of the theoretical capacity of their podia

As mentioned earlier this respiratory inefficiency is probably related to the poorly developed mechanisms for movement of external and internal media The movement of the circulatory fluid as effected by cilia is less efficient than that caused by the vertebrate heart In the sea urchin there are no well developed distributing vessels and capillaries There is thus a relatively inefficient mechanism for distribution of oxygenated fluid to the tissues and for deoxygenated fluid back to the podia The fact that fluid inside the same ampullae or podium moves simultaneously in opposite directions must certainly contribute to render the system inefficient due to cross diffusion and fluid mixing Moreover the eel blood contains hemoglobin whereas the circulatory fluid of sea urchins have the oxygen capacity of water only The advantages of this 20-fold difference in oxygen capacity together with the evolution of an adequate circulatory system are manifest They have reduced the energy needed by the fish to achieve gas exchange and they have enabled the fish to reduce its exchange area with the surrounding milieu This latter factor may have contributed to the development of an internal osmotic environment different from the external one thus making the *organism partially independent of one element of its environment*

I wish to acknowledge excellent assistance from Mrs J Haivie Cand real F Walvig of the Biological Station of Drobak very kindly collected the material The investigation was supported by The Norwegian Research Council for Science and the Humanities and Norsk Varkrigsforsikrings Fond

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STEEN J B and O IVERSEN Modernized Scholander respirometer for small aquatic animal *Acta physiol scand* 1965 *63* 171 –174

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From the Institute of Zoophysiology University of Oslo Blindern Norway

Modernized Scholander Respirometer for Small Aquatic Animals

By

J B Steen and O Iversen

Received 1 June 1964

Abstract

Steen J B and O Iversen *Modernised Scholander respirometer for small aquatic animals* Acta physiol scand 1965 *63* 171—174 — A method is described for measuring oxygen uptake of small aquatic animals using from 0 1 to 20 ml oxygen/hr The water containing the animal is continuously equilibrated with air in a closed system and respiration is measured as the oxygen necessary to maintain a constant gas volume in the presence of a carbon dioxide absorber

The oxygen consumption of an animal living in water can be determined in the gas phase of a closed system provided the water is thoroughly equilibrated with the gas phase The respirometer to be described in the following was developed for studying respiration in sea urchins (Steen 1964) It is based on the same principle as that employed by Scholander (1949) The present modification is however more rugged reliable and simpler to operate than the original version The respirometer permits measurements over a wide range of oxygen consumption Several ones can easily be arranged in series

Principle

The animal is enclosed in a respiration chamber where the water is continually equilibrated with the air above it The gas phase is separated from a compensation chamber by a fluid manometer and is connected with an oxygen filled syringe and a carbon dioxide absorber The oxygen consumed by the animal is replaced by oxygen from the syringe to maintain the manometer meniscus at a certain position Oxygen consumption is therefore read directly as the difference in syringe volume over suitable periods of time

Apparatus

The apparatus has the following parts (Fig 1) Manometer block glued to the lids of a respiration chamber and a compensation chamber rubber sealing rings closing frames motordriven magnet for driving a stirring paddle at the water surface carbon dioxide absorber oxygen syringe and temperature-regulated water bath

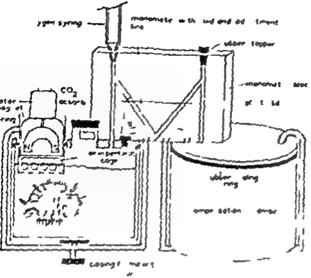

Fig. 1. Drawing of respirometer.

The top edge of the chamber should be well ground to ensure a tight seal when the flat 1 mm thick rubber sealing ring is pressed between it and the plastic lid. Fint Mason jars may be used. Each chamber is closed by a brass frame screwed to bolts against a 3-cm wooden disk underneath. The lids for both chambers are glued to a plastic manometer block furnished with a flange by which the unit is screwed to the supporting bank (Fig. 2). Two such banks each holding 2 and 3 [illegible] ml units respectively are [illegible] tionally fastened in 60 [illegible] 30-cm bath. The vacant place may be used for a flask containing salt water being equilibrated with the desired gas mixture in advance [illegible] between experiments.

The stirring unit consists of two parts (Fig. 1). One is a plastic [illegible] enclosed in a circular plastic housing which has circular holes [illegible] diameter in its wall and floor but has intact roof. This housing is fastened [illegible] the lid of the respiration chamber which is fitted with an O ring to make an air tight seal. The wall of the stirrer cylinder forms a chimney extending above the surface of the lid so that the water level of the surrounding bath may [illegible] over the lid. The vertical position [illegible] unit may be varied. For a chamber containing 400 ml water and 30 ml gas [illegible] will extend about 1 cm above the surface of the lid. The second part of the stirring unit is an ESCAP SR [illegible] motor for [illegible] V DC current with an ESC [illegible] gear (Fig. 2).

The heat produced by this motor is too small to affect the [illegible] of the respiration [illegible] syringe measurably. It is mounted on a plastic cylinder which fits into the [illegible] of the stirrer cylinder and in which the magnet rotates shielded from the [illegible] splashes of water.

Ascarite serves as carbon dioxide absorber. It is held in a plastic [illegible] a rubber stopper closing the 2-cm high absorber chimney.

Any manometer fluid may be used. The oxygen is [illegible] the [illegible] block by forcing the syringe [illegible] by a [illegible] rubber [illegible] its tapered hole.

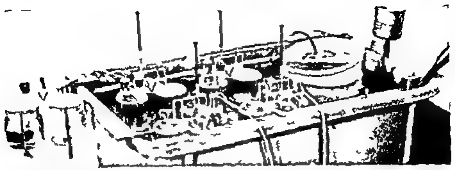

Fig 2 Photograph of set up with five resp rometers To the left is seen a resp rometer fastened to the side arm to the far right is seen the stirrer and the bucket with water being equilibrated Four respirometers and one vacant place is seen in the bath

Operation

The unit is first screwed on to a side arm of the supporting rack extending outside the bath (Fig 2) The respiration chamber is filled with equilibrated water from the bucket in the water bath (Fig 2) The animal is put into the chamber which is closed by means of the tightening frame

The unit is then transferred to the support in the water bath To protect the motor it is wise to disconnect and remove it during this procedure By inserting a pre determined length of suction tubing into the carbon dioxide chimney the water level is adjusted down to a height giving the maximum equilibration efficiency (i e the best compromise between stirring and whipping) The motor unit is put in position and connected to a 4—5 V battery If experiments are to be conducted with air equilibrated water the carbon dioxide chimney is left open for some minutes while the stirrer works Then the rubber stopper is inserted to close the compensation chamber and the carbon dioxide absorber is fastened in the chimney The syringe is filled with oxygen fitted to the animal side of the manometer block and its top sealed with a drop of parafin oil

At intervals the volume of oxygen needed to reset the manometer meniscus to its mark is recorded The volume must be corrected to standard condition using the temperature present in the syringe not in the bath and the barometric pressure at the moment the compensation chamber was closed For this reason one should use small syringes for oxygen refill and care should be taken that the room temperature be as constant as possible

The present design appears to have several advantages over the original one proposed by Scholander (1949) Each unit can be served separately and the chambers are sealed by a metal frame thus not requiring a rubber stopper to-glass connection which may slip In addition this stirrer unit is more compact with no possibilities of leakage (in the old edition grease from the capillary frequently centered the animal chamber) We also find it convenient that the carbon dioxide absorber may be removed or introduced without disconnecting the manometer block An additional injection stopper makes it possible to introduce substances (ex poisons hormones) during experiments as well as removing gas or fluid samples for analysis When the motor unit is removed the rest of the unit can be washed as any other piece of plastic and glass The present version has been used for continuous 24 hour experiments and has proven very reliable during daily routine work

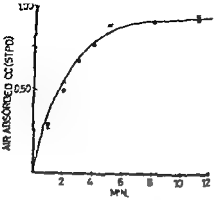

Fig 3 Absorption of air into 500 ml of 90 air-saturated water at 17 C. Two parallel series of measurements are shown According to the solubility coefficient for air in water (1 975 ml 100 ml at 17 C) 0 99 ml air should have been absorbed. The somewhat smaller measured absorption of 0 91 ml is due to incomplete deaeration of the water

The respirometer may conveniently be used also to measure oxygen uptake of small airbreathing animals.

Accuracy

Blank runs indicate that with 500 ml aerated water the system is stable to within 0 01 ml per hour as measured with a 1-cc tuberculine syringe Analysis of the oxygen content (Scholander *et al* 1955) in air-equilibrated water of the respirometer during experiments showed that the oxygen content was within 2 [illegible] of the theoretical value During the experiments with sea urchins we sometimes obtained a positive readin from water in which an animal had stayed for hours due to eggs sperm and pieces of algae ejected by it and to the microfauna living on its surface Careful cleaning of the respiration chambers between expriments is therefore recommended

The efficiency of equilibration was tested as described by Scholander 1942 by sub[illegible] an equal amount of deaerated water for water in the completely equilibrated [illegible] and recording the time required for restabilization As shown in Fig 3 complete [e]quilibration is reached within 8 min The volume of air taken up agrees closely with [t]he volume expected from the air solubility in water at that temperatur[e]

From Fig 3 it can be seen that the initial rate of air absorption in 90 [illegible] saturated water is 0 60 ml min Due to th[e] solubilities of oxygen and nitrogen in water about 34 % of this air would be oxygen Thus about 0 20 ml oxygen is absorbed per min which is probably plenty for any animal that can be fitted into the respirom[e]ter

The efficiency of CO_2 absorption was tested by injecting 1 ml 100 [illegible] CO_2 int[o] the gas phase through the injection stopper while compensating with the syringe [illegible] of the CO_2 was removed within 3 min

The respirometers are commercially available from Mr O Iversen at th[e] Institut of Zoophysiology University of Oslo Blindern Norway

We wish to acknowledge the enthusiastic assistance of Mrs J Haasie and the technical [illegible] of Mr O Brynhildsrud and Mr B Savalov The investigation was supported by Norsk Var[illegible] [illegible] Fond and by The Norwegian Research Council for Science and the Hu[illegible]

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STEEN J B Comparative aspects of the respiratory gas exchange [illegible] *physiol scand* [illegible]

Acta physiol scand 1965 63 175—185

From the Institute of Physiology University of Uppsala Sweden

The Transmucosal Migration of Water and Hydrogen Ions in the Stomach

By

KARL JOHAN ÖBRINK and MARGARETA WALLER

Received 6 June 1964

Abstract

Öbrink K J and M Waller *The transmucosal migration of water and hydrogen ions in the stomach* Acta physiol scand 1965 *63* 175—185 — A preparation consisting of an isolated flap of the ventral wall of a cat's stomach with an intact blood supply was mounted between two perspex chambers The transmucosal potential was measured The mucosa was electrically shortcircuited and the current registered. A mixture of HCl and Na_2SO_4 was instilled in the mucosal chamber and the disappearance rates for the hydrogen and sulphate ions were determined The quotient between these two (Qm) was calculated. No stimulation to secretion of HCl was given but a complete secretory rest could not be garanteed A slight secretory activity tended to give too low values of Qm The highest values of Qm coincided with a similar quotient for a cellophane membrane (Qc) which was considered to indicate that hydrogen ions disappeared through the gastric mucosa in the same way as did the sulphate ions which were thought to migrate by way of an intercellular diffusion The results were thought to support the diffusion hypothesis of Teorell Similar experiments with tritiated water suggested that 75 per cent of the water migration is transcellular

It is well known that the hydrogen ion concentration of an acid solution instilled in a non secreting stomach decreases exponentially (Teorell 1933 Öbrink 1948) Teorell described this decrease as analogous to a diffusion process but this formal way of presenting the events is of course no proof for diffusion Later other authors adopted the diffusion theory (cf Friedman 1951 1952 There are however other possibilities for a decrease in [H] e g a neutralization with ammonium formed by the gastric urease from urea (Fitzgerald 1946 1950 Fitzgerald and Murphy 1948

In addition there is the well known two-component hypothesis of Hollander's (1938) which attributes different low H -concentrations to different degrees of admixture with a non acid component of the gastric juice possibly containing bicarbonate

In the last 30 years acidity regulation has been ascribed to many factors The fact that the permeability coefficient for H in a nonsecreting stomach can account completely for the acidity secretion rate relation in a histamine stimulated pouch

(Öbrink 1948) has been taken as evidence that diffusion of hydrogen ions through the gastric mucosa is a main factor in acidity regulation. However more experimental evidence should be presented for the "diffusion hypothesis" before results from diffusion formulas can be safely applied to actual biophysical processes.

The present paper presents further evidence for the existence of significant H ion diffusion through the gastric mucosa.

Methods

Cats were used. They had been starved for 12—18 hours. Anaesthesia was induced with chloroform and then chloralose urethane (1 : 10) was given intravenously.

Surgical procedure. A midline incision was made. The pylorus was ligated and divided. All vessels supplying the greater curvature were ligated as well as the a. ventriculi dorsalis. V. coronaria ventriculi which passes along the lesser curvature and joins the portal vein shortly after passing the pylorus was dissected free.

The a. gastrica sinistra was dissected free together with v. gastrolienalis accompanying it. The branches from these vessels supplying the cranial part of the stomach were ligated and cut. Some small vessels of the a. lienalis going to the stomach were divided. Finally the oesophagus was ligated and the stomach cut free.

Now the middle and caudal part of the ventral surface of the stomach was the only part supplied with blood.

The stomach was opened along the greater curvature and fixed in the lucite chamber in Fig. 1.

The chamber was fixed with the mucosa in a vertical position. The vessels [illegible] left the chamber (o) through an opening that was tightened by placing cotton wool around the vessels very carefully so that no stasis was produced, and then by covering the cotton wool with vaseline. This procedure gave a water tight through way for the vessel without interfering with the blood flow.

[illegible] procedure

[illegible] serosal part of the chamber (o) was filled with saline at body temperature th[illegible] [illegible] tube in the top (m). Th[illegible] tube was connected to a temperature regulated [illegible] reservoir (about 3 l). By means of a S[illegible] pump the saline was circulated through th[illegible] system. The return tube kept the saline at a constant level in the chamber. The temperature was regulated by changing the rate of flow and was controlled by a termistor.

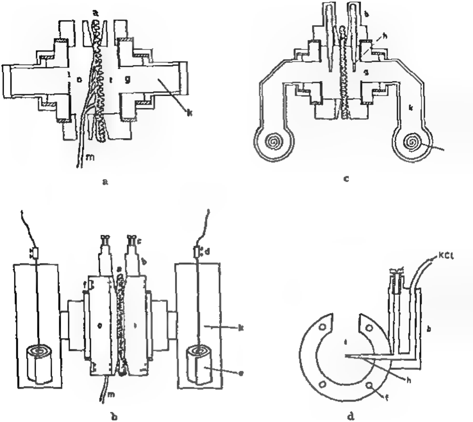
To keep the saline concentration at a constant level a conductometric relay operated an electric stop-cock which delivered distilled water to the saline system whenever the conductivity increased due to evaporation.

The gross appearance of the stomach sheet could be observed through the lucite w[illegible]. Pulsations and colour were in all experiments controlled. During the experiments no stimulation for gastric secretion was given.

The mucosal chamber [illegible] was filled with saline until the mucosa was in good [illegible] recovered from the surgery, usually 1—3 hours. A test solution was then [illegible] [illegible] varied from [illegible] to [illegible] ml depending on the thickness and degree of [illegible] of the mucosa. [illegible] 0.1 ml samples were pipetted from the test solution every 10 or 30 min for analysis. [illegible] the pulsations in the mucosa and its [illegible] [illegible] by an extra [illegible] in the [illegible] chamber was considered not necessary.

After termination of the experiment the cat was given about 0.1—0.5 [illegible] hydrochloride s.c. The mucosal chamber [illegible] was emptied and the secretion of HCl [illegible]. In all experiments with successful operations the stomachs were able to secrete HCl even after many hours in the chamber.

Transmucosal potential was measured with two calomel electrodes connected [illegible] chambers with saturated KCl-bridges through (b) and (b). See Fig. 1 [illegible] and [illegible] KCl was [illegible] [illegible] to the top of (b) by the screw (c). The potential was registered on a Grass polygraph with preamplifier. The potential was usually low immediately after the operation but rose to a steady level in about one to 3 hrs.



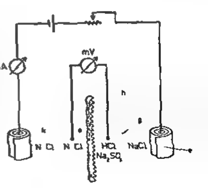


Fig 1 *The persp x chamber with the intact flap of „ stric mucosa*
a) A vert cal cross section of the chamb r (a) = the gastr c wall (m) = blood ves els (o) = out side chamber (s rosal s de) (i) = nside chamber (mucosal side) (g) = a thick multilayer cellophane membrane s parating the test solution n (i) and the sal ne n (o) from the saline in (k)
b) A side view of the chamber (f) = one of 4 screws keeping the 2 halves of the chambe tog th er In the figure are seen th two big silv silv chlor de electrodes (e) that were used for the short c rcuit g current f om a batters connect d to the electrodes at (d) For (b) and (c) see F g 1 d
c) A top view of th chamber Two plasti tubes (h) re seen ext nd ng into the chambers They conta n satur ted KCl solut on
d) S de view of the KCl bridge connected through (b) with a calomel lect ode With the screw (c th KCl could be arefully moved exactly to the tip of (h)
e) A schematic v ew of th electr cal c rcuit ng In some experim nts the content in (i) was changed to HCl n tritiated water

In the case of HCl instilled the initial conditions will be $H = H_o$ for $t = 0$. This gives

$$H = \frac{C_o}{1 - \frac{k}{v}} \left[1 - \frac{1 - \frac{H_o}{C_o}\left(1 - \frac{k}{v}\right)}{\left(1 - \frac{vt}{p}\right)^{\left(1 - \frac{k}{v}\right)}} \right] \qquad (6)$$

This is a generalized equation containing several special cases.

Case 1. $p = 0$ gives

$$H = \frac{C_o}{1 - \frac{k}{v}} \qquad (7)$$

This describes the well known hyperbolic relationship between the acidity and the secretion rate. Note that t has disappeared from the equation which means that the acidity will be the same irrespective of the time the juice remains in the stomach, i.e. whether the juice is running freely from the stomach or withdrawn at regular or irregular intervals.

Case 2. $H_o = 0$

This is the case treated by Teorell (1947) and gives

$$H = \frac{C_o}{1 - \frac{k}{v}} \left[1 - \frac{1}{\left(1 - \frac{vt}{p}\right)^{\left(1 - \frac{k}{v}\right)}} \right] \qquad (8)$$

Also this equation is reduced to (7) when $p = 0$ or $t = \infty$.

Case 3. $t = 0$ gives

$$H = H_o$$

which of course follows from the initial conditions.

Case 4. $t = \infty$ gives

$$H = \frac{C_o}{1 - \frac{k}{v}} \qquad (7)$$

This is the same as $p = 0$ because when $t = \infty$ the influence of H_o has completely disappeared.

Case 5. $v = 0$

This is the condition best suited for diffusion studies. The transformation of (6) in this case may perhaps need a more detailed description.

Eq. (6) can be rewritten as

$$H = \frac{C_o}{1 - \frac{k}{v}} - \frac{\frac{C_o}{1 - \frac{k}{v}}}{\left(1 - \frac{vt}{p}\right)^{1 - \frac{k}{v}}} + \frac{H_o}{\left(1 - \frac{vt}{p}\right)^{1 - \frac{k}{v}}} \qquad (9)$$

For $v = 0$ Eq. (9) gives

$$H = 0 - 0 + \lim_{v \to 0} \frac{H_o}{\left(1 - \frac{vt}{p}\right)^{1 - \frac{k}{v}}} \qquad (10)$$

or

$$\frac{H}{H_o} = \lim_{v \to 0} \left(1 - \frac{vt}{p}\right) \cdot \lim_{v \to 0} \frac{1}{\left(1 - \frac{vt}{p}\right)^{-\frac{k}{v}}} = 1 \cdot \lim_{v \to \infty} \frac{1}{\left(1 - \frac{kt}{v}\right)^{k}} \qquad (11)$$

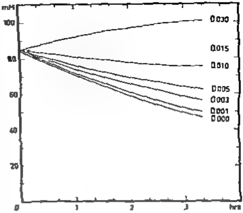


Fig. 2. *The expected change in hydrogen ion concentration in the mucosal chamber.* An 85 mM HCl solution was instilled. The permeability coefficient was assumed to be 0.015 ml/min and the primary acidity to be 170 mM. The H concentration will change differently with different secretion rates v of the stomach. At a rate of 0.015 ml/min no change in concentration can be expected. Only for v = 0.000 ml/min will a simple exponential curve be obtained. The general appearance will follow equation (6) (see text).

According to the fact that

$$\lim_{n \to \infty} \left(1 + \frac{x}{n}\right) = e$$

eq. (11) turns out to write

$$\frac{H}{H} = e \; \frac{k}{p}$$

or

$$H = H_0 e \; \frac{kt}{p}$$

which was our equation (1).

From the above theoretical deductions it is evident that whenever $v \neq 0$ the acidity in an instilled volume will not follow the simple equation (1) but rather the more complicated eq. (6).

The consequences of the *interference from a concomitant secretion* on the acidity in an instillation experiment can be seen in Fig. 2. Here the parameters in eq. (6) have been assumed to have the following values: $C_0 = 170$ mM, $H = 85$ mM and $k = 0.015$ ml/min. Different values for v were inserted in eq. (6) and $H = f(t)$ determined. For only $v = 0.000$ could the simple exponential fall in acidity be expected. If the other curves were treated as if $v = 0$ then a too low k value would be calculated. In the particular case when $v = k$ the acidity would never change but remain at the H_0 level. Generally it should be expected that the acidity would not change when $v = \frac{k}{a-1}$ where $a = \frac{C}{H_0}$. In the figure $a = 2$, which means no acidity change when $v = k$. If $a = 3$ ($H \approx 56$ mM) similar conditions would occur for $v = k/2$. That means that the lower the H the more important will the influence of the secretion be. If $v > \frac{k}{a-1}$ then the acidity will even increase above the H_0 (see fig. 2).

The error in determining the permeability coefficient k will thus tend to decrease the value. If two or more experiments are performed on the same stomach the highest k value will probably be the most correct one if the stomach was not at a complete secretory rest.

Results

Transmucosal potential and short-circuiting current

The potentials immediately after the surgery were usually low but increased during the next couple of hours. The saline in the mucosal chamber was then changed for

TABLE II. PD in the gastric mucosa

NaCl in the mucosal chamber		HCl in the mucosal chamber		Approx. short circuiting currents ($\mu A/cm^2$)
At the start of the exp.	After recovery	After instillation of HCl	At the end of the exp.	
8	20 (205 min)	37	36	103–9[illegible]
10	26 (135 min)	40	42	141–9[illegible]
15	42 (95 min)	54	58	197
20	32 (30 min)	40	54	211–183
44	60 (45 min)	62	65	239–204
10	36 (1[illegible]0 min)	48	36	
12	42 (60 min)	53	46	11
20	52 (45 min)	62 (64)	60	—

TABLE III

The quotient k_H/k_{SO_4} in experiments with

Cellophane membrane (Qc)	Gastric mucosa (Qm)
3.61	[illegible].50
3.50	3.48
2.99	1.91
3.36	3.19
3.41	2.44
3.08 (not shortcircuited)	3.03
3.04 (not shortcircuited)	2.15
Qc = 3.28	[illegible]

the test solution. This immediately increased the potential difference due to the addition of a diffusion potential created by the HCl. The potentials and the shortcircuiting currents from the HCl-sulphate instillation experiments are shown in Table II.

Comparison of transport rates for H^+ and SO_4^{--} ions

A solution of about 85 mM of HCl and 40 mM of Na_2SO_4 was instilled in the diffusion chamber. The result from an experiment with a gastric mucosa is seen in Fig. 3. It is noticed the faster decrease in acidity compared to sulphate disappearance. The k_H/k_{SO_4} from the mucosa experiments (Qm) as well as those with a cellophane membrane (Qc) are collected in Table III. There it is seen that the Qc is somewhat scattered around a mean value of 3.3. The Qm showed a greater deviation but had some values in the same region as Qc (above or equal to 3.0). The chloride concentration was in

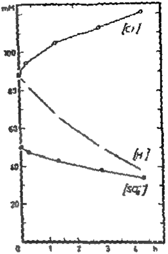


Fig 3 A typical experiment with instillation of a mixture of 85 mM HCl and 50 mM Na_2SO_4 in the mucosal chamber

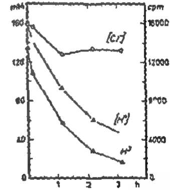


Fig 4 A typical experiment with instillation of a 170 mM HCl solution containing tritiated water

Fig 3) rose towards a steady state value that was significantly higher than that of plasma (110 mM) This phenomenon has been discussed elsewhere (cf Heinz and Öbrink 1953)

Comparison of transport rates for H and HTO

In identical types of experiments the disappearance rates of H ions and tritiated water (HTO) were compared The results from a typical experiment are shown in Fig 4 170 mM HCl containing 13 000 cpm H^3 was introduced in the mucosal chamber in experiments both with a gastric mucosa and with a cellophane membrane. Table IV gives the collected results Qm averaged 7 3 while the corresponding Qc was 0 59

TABLE IV

The quotient k_{HTO}/k_{H} in experiments with

Cellophane membrane (Qc)		Gastric mucosa (Qm)		Whole stomach (not shortcircuited)
0.52	(not shortcircuited)	1.79		2.86
0.65	(not shortcircuited)	1.58		2.57
0.66	(not shortcircuited)	2.34		2.14
0.67	(not shortcircuited)	2.67		2.45
0.63	= Average	3.37		2.48
		2.40		2.17
0.62		2.20	(not shortcircuited)	1.66
0.57		2.20		2.32
0.60				1.88
0.56				1.87
				2.1
0.59	= Average	2.31	= Average	2.23 = Average

Discussion

The permeability coefficients (k) for hydrogen and sulphate ions have been compared in isolated and electrically shortcircuited gastric mucosa membranes in vivo. If no driving forces other than the chemical potential gradients were responsible for the disappearance of these ions from an instillate and if the resistances to ion movement were the same for both ions one would expect the permeability coefficients to be proportional to ionic mobilities. The ionic mobilities in very dilute solutions at 25° C have been calculated to 36.2×10 cm/sec for hydrogen ions and 8.27×10 cm/sec for sulphate ions, i. e. a ratio k_{H}/k_{SO_4} 4.4. For higher temperatures this ratio will decrease somewhat. For less dilute solutions the mobility figures cannot however be safely used. In electrolyte mixtures like in the present experiments the relation between the two permeability coefficients will be even more unpredictable.

The reason is that in a complex system like the present one the fluxes of the different ions interfere with one another. The degree of interference depends on several factors such as concentration, size of molecules or ions in the solution and pH (e. g. affecting the fixed charges of the membrane). It is therefore safer not to rely upon the above given mobility figures but to compare systems as identical as possible but with different separating membranes (stomach mucosa and cellophane membrane).

As was shown in Table III the k_{H}/k_{SO_4} for the cellophane membrane (Qc) was about 3.3 whereas the same quotient for the mucosa (Qm) was as an average lower than Qc. As was discussed above (Theoretical p. 7) the errors in determining k_{H} in the stomach tended to give too low values. The highest values should therefore be considered to be the most reliable ones.

In a non-shortcircuited gastric mucosa the electrical potential ought to enhance the flux of SO_4 ions but retard that of H ions. In the shortcircuiting experiments

there was no garantee that the current density at the membrane surface was absolutly uniform. It could therefore happen that only the middle portion where the trans mucosal potential was recorded was brought to zero potential whereas the periphery of the membrane was only partly shortcircuited. In this case the k_H would again be somewhat too low and the $k_{SO_4}{}^{-}$ too high resulting in too low Q values.

The highest values of Qm were in fact almost equal to the Qc which seams to indicate that the disappearance of H from the stomach lumen depends on an intercellular diffusion (SO is considered to remain exclusively extracellular). If so one could explain the disappearance of H from the gastric juice as a diffusion process as was proposed in 1933 by Teorell.

The second finding that k_{HTO}/k_H for the stomach was higher (2 3) than for the cellophane membrane (0 59) showed that the penetration of water was highest in the mucosa. As no hydrostatic pressure gradient was present (especially not in the direction lumen blood) and as no marked changes in the volume of the mucosal chamber occurred it seems as if the diffusion area for HTO was 2 3/0 59 = 4 times larger in the mucosa compared to that in the artificial membrane. If the above conclusion of *inter*cellular diffusion of H ions is correct it points to the probability that 3/4 of the water flux took place in a *trans*cellular route and only 1/4 in an *inter*cellular one. This is perhaps not surprising but is of importance in the discussion of the origin of the volume secretion accompanying the output of HCl.

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Further Studies of the Thyroidal Response to Local Cooling of the "Heat Loss Center"

By

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Abstract

Andersson B A H Brook and L Ekman *Further studies of the thyroidal response to local cooling of the heat loss center* Acta physiol scand 1965 63 186 192 The rise in plasma protein bound I^{131} (PBI^{131}) which occurs during local cooling of the preoptic anterior hypothalamic region in the goat was found to be accompanied by a corresponding rise in total serum protein bound iodine (PBI) confirming that such cooling may cause a conspicuous increase in thyroid hormone secretion Experiments involving graded central cooling revealed that moderate cooling of the preoptic anterior hypothalamic region was sufficient to elicit the full thyroidal response whereas deeper cooling in certain circumstances may be completely ineffective This suggests that the thyroidal response to such cooling reflects a true physiological mechanism operating already at a moderate general hypothermia Local cooling of the heat loss center with the animal in a hot environment revealed that the thyroidal response is obtained even in the absence of peripheral cold inflow

It was previously shown that local cooling of the preoptic anterior hypothalamic region (the heat loss center of Magoun *et al* 1938) caused an often *conspicuous rise* in plasma protein bound I^{131} (PBI^{131}) in goats pre treated with radioiodine This thyroidal *response to central cooling* was temporarily blocked by the administration of thyroxine and was permanently abolished after lesions of the median eminence (Andersson *et al* 1963 a and b) During local cooling of the heat loss center there was also an increase in urinary excretion of adrenaline and noradrenaline (Andersson *et al* 1963) In these studies it was further observed that local warming of the same part of the brain had the reverse effect *i e* inhibited the thyroidal and the sympathico adreno medullary responses to a general cold stress It was therefore suggested that central warmth detectors in the rostral hypothalamus not only serve to mobilize physical heat loss mecha

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nisms during hyperthermia, but also exert a brake on both neurogenic and hormonal cold defence mechanisms at normal brain temperatures (Andersson Gale and Hokfelt 1964)

In the previous studies local cooling of the "heat loss center was in most animals found to cause a very marked rise in plasma PBI[131] In a few goats however the rise in plasma PBI[131] was small It therefore seemed to be of interest to determine whether the rise in plasma PBI[131] during central cooling really mirrors the release of hormone from the thyroid gland, and whether variations in the intensity of cooling or minor changes in the placement of the thermodes would influence the plasma PBI[131] response Finally the heat loss center of a goat placed in a hot environment was cooled to find out if a thyroidal response would be obtained also in the absence of peripheral cold inflow

Methods

The experimental animals comprised three adult female goats (A B and C) weighing from 35 to 42 kg The animals had gold plated silver thermodes for local brain cooling permanently implanted medially in the preoptic anterior hypothalamic region as described earlier (Andersson et al 1963 a) The intensity of cooling was determined with thermocouples implanted close to the lateral surface of the thermodes

Care and management of the animals The goats were routinely maintained in metabolism cages in an animal room at a temperature of 20 ± 3 C. All experiments were done in this their normal position and customary environment. Only during central cooling in a hot environment (36 ± 1 C) was goat A moved into an adjacent room

Technique of central cooling In the experiment designed to test the influence of a hot environment and in some of the experiments performed to compare the plasma PBI and serum PBI responses central cooling was performed as in earlier experiments (Andersson et al 1963 a) i e by perfusion of the thermodes with iced water at a constant high speed Cooling in this manner lowered the brain temperature 1 mm lateral to the surface of the thermode to about 25 C This more intense central cooling will be referred to as deep central cooling In the studies involving graded central cooling the brain temperature 1 mm lateral to the surface of the thermode was maintained close to 35 31 and 35 C respectively The less intense brain cooling was obtained by using water at 22 C and by reducing the perfusion rate

Studies of thyroid activity At the beginning of each experimental period the goats were given 60 or 100 microcuries of I (as carrier free NaI) by stomach tube Thyroidal uptake of radioiodine total plasma I[131] and plasma PBI[131] were determined as in earlier experiments (Andersson et al 1963 a) Serum PBI was determined on duplicate samples according to the method of Barker and Humphrey (1950)

The TSH preparation used in the present study was Actyron Ferring

At the conclusion of the studies on endogenous thyroid hormone release all goats were surgically thyroidectomized

Results

I Plasma PBI[131] and serum PBI levels

In the three goats parallel estimations of plasma PBI[131] and serum PBI were made before during and after two hour periods of local cooling of the heat loss center The pre-cooling samples and the samples taken 24 hours after central cooling showed very stable serum PBI values mean 4 2 µg 100 ml d 0 2 n 9 In all animals there was a good correlation between the rise in plasma PBI[131] and the rise in serum PBI ob-

Grateful acknowledgement is made to D Westdijk M D Head of the Central Laboratory Danderyds Sjukhus Danderyd by whose courtesy the serum PBI determinations were made

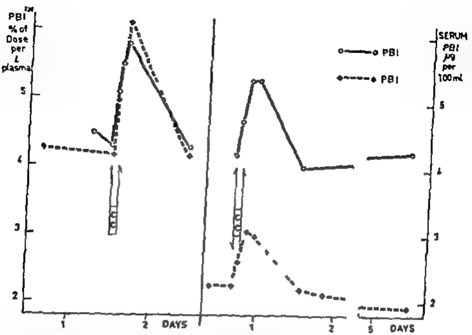

Fig 1 A comparison between plasma PBI[131] and serum PBI responses to local cooling of the heat loss center of goat A (left) and goat B (right) The similarity of the curves confirms that such cooling can cause a marked rise in thyroid hormone secretion
CC = 2 hours of central cooling

tained during and just after two hours of central cooling The average increase was 37 % for plasma PBI[131] and 35 % for serum PBI The mean of the peak levels of serum PBI found within an hour after termination of central cooling in the three goats was 5 7 ± 0 2 µg/100 ml Fig 1 shows the close parallel between the plasma PBI[131] and the serum PBI levels during experiments performed in goats A and B

During the first week after surgical removal of the thyroid gland the disappearance rates of plasma PBI[131] and of serum PBI were similar

II Influence of graded central cooling and slight differences in the placement of the thermodes

All goats used in this study had a high thyroid uptake of I[131] and their plasma PBI[131] levels three days after the administration of radioiodine ranged from 2 to 4 per cent of the given dose per liter of plasma The only apparent difference in the experimental conditions between the three goats was the position of the thermodes within the pre optic/anterior hypothalamic region Goats A and B had their thermodes placed mainly in the preoptic region i e above and in front of the optic chiasm The thermode in goat C partially overlapped the position of the thermodes in the two other animals but extended further back into the hypothalamus proper as shown in Fig 2

The goats were all initially subjected to two hour periods of deep central cooling This caused a marked rise in plasma PBI[131] in goats A and B but no rise at all in goat C Subsequent intravenous injection of 1 I U of TSH (Actyron Ferring) to goat C did however produce a marked thyroidal response Fig 3 similar to that obtained

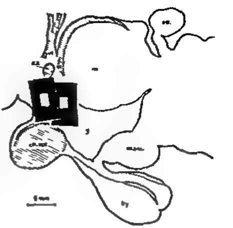

Fig 2 A drawing of a midline sagittal section through the diencephalon of the goat to show the different thermode placements in the goats used in the present study Note that whereas the thermodes in goats A and B were placed almost exclusively in the preoptic region the thermode in goat C extended further back into the anterior hypothalamus Goat C responded to moderate central cooling with a marked thyroid activation whereas deep central cooling had no such effect (Fig 4 left) In goats A and B both deep and moderate central cooling caused marked thyroid activation.
c. a = anterior commissure — ch opt = optic chiasm — ep = epiphysis — hyp. = hypophysis — mam. = mammillary body — m. i = massa intermedia — 3 = the third ventricle

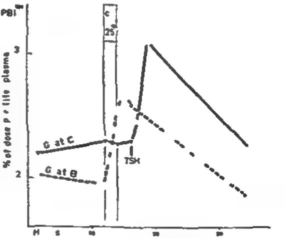

Fig 3 Difference between "deep central cooling" in goat B (thermode almost exclusively in the preoptic region) and goat C (thermode extending back into the hypothalamus proper) Note the characteristic thyroid response in goat B and the lack of response in goat C. The ability of the thyroid of goat C to respond to injected TSH indicates that no thyrotrophic hormone was released during "deep central cooling" in this animal.
CC = 2 hours of central cooling Brain temperature 25 C 1 mm lateral to the thermode
TSH = Intravenous injection of 1 IU of TSH (Actyron Ferring)

later in the same goat during moderate central cooling All animals were then subjected to milder central cooling Repeated experiments in goat C showed that moderate cooling (brain temp 31 and 35 C, 1 mm lateral to the thermode) caused a marked thyroidal response whereas "deep central cooling (brain temp 25 C) never had any thyroidal effect (Fig 4 left) A reduction in the intensity of central cooling by 10 C in goats A and B gave approximately the same thyroidal response as deep central cooling (Fig 4 right)

III Thyroidal response to central cooling in a hot environment

Goat A was used to determine if a thyroidal response to central cooling would also be obtained in a hot environment. Ten minutes before the start of central cooling the goat was moved into a room heated to 36 ± 1 C. and subjected to 80 min of deep central

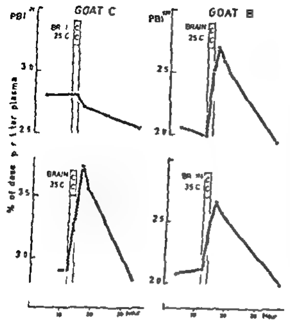

Fig 4 A comparison of deep (Brain 25 C) and moderate (Brain 35 C) central cooling in two goats with differently placed thermodes Goat C had its thermode placed partially in the preoptic region and partially in the hypothalamus proper The thermode in goat B was situated almost entirely in the preoptic region (Fig 2) Note that in goat B both deep and moderate cooling elicited a thyroidal response whereas in goat C only moderate cooling was effective
Brain temperature was recorded 1 mm lateral to the thermodes
CC = 2 hours of central cooling

cooling During this period the goat developed a general core hyperthermia of 41 6 C The experiment had to be discontinued at this stage to avoid a further dangerous rise in body temperature During the 80 min period of local cooling of the heat loss center the plasma PBI[131] increased by 40 per cent In three previous experiments performed at a room temperature of 19 C the plasma PBI[131] of goat A had increased by 40 35 and 60 per cent respectively after the same period of deep central cooling

Discussion

Present knowledge of nervous control of endocrine activity derives largely from the fundamental studies of Harris and coworkers cf Harris 1955 who showed that nervous stimuli influence the secretion of pituitary trophic hormones via the hypothalamus and then via a humoral link transported in the hypothalamo-hypophyseal portal vessels Such a mechanism is apparently responsible for the accelerated release of TSH in the cold since an increased secretion of thyroid hormone upon cold exposure no longer occurs in animals with the anterior pituitary transplanted into the eye (C v Euler and Holmgren 1956)

Electrical stimulation Harris and Woods 1958 and various lesion experiments cf Purves 1964 have revealed that the anterior hypothalamus controls the secretion of TSH from the adenohypophysis As pointed out by Harris and Woods 1958 it may however be inadvisable to consider the anterior hypothalamus a center regulating TSH secretion since it most likely represents a neurohumoral mechanism akin to a final common path by which other parts of the central nervous system may influence the secretion of TSH Harris and Woods mention the k that the adjacent preoptic at

loss center (Magoun *et al* 1938) may influence the secretion of TSH via this final common path Direct evidence that such is really the case was provided by experiments involving local cooling and warming of the preoptic/anterior hypothalamic region of unanaesthetized goats (Andersson *et al* 1963 a and b) In these studies the plasma PBI[131] level was taken as the index of hormone release from the thyroid gland Although the plasma PBI[131] level in most cases is a fairly sensitive index of thyroid hormone secretion (cf Goolden 1964) it could not be taken for granted that such was the case in the goat under the particular experimental conditions The present study has shown that the rise in plasma PBI[131] obtained during and just after central cooling is accompanied by a corresponding rise in total serum PBI and has therefore demonstrated beyond doubt that local cooling of the heat loss center may cause a marked increase in the secretion of hormone from the thyroid gland

In the previous experiments (Andersson *et al* 1963 a) rather intense central cooling was used as the experimental tool In animals having a more posterior placement of the thermode this may have considerably lowered the temperature not only of the preoptic region, but also of parts of the hypothalamus concerned with the release of neurohumor stimulating the adenohypophysis to secrete TSH It might therefore be argued that the thyroidal effect of central cooling could be secondary to a nonspecific hypothermia induced release of neurohumor into the hypothalamo-hypophyseal portal vessels The present experiments involving graded central cooling indicate that such is not the case Rather it seems as if deep central cooling involving not only the preoptic region but also the anterior hypothalamus blocks the TSH releasing mechanism Goat C which had a thermode extending posteriorly into the hypothalamus to the level of the paraventricular nuclei (Fig 2) thus responded to moderate central cooling with marked thyroidal activation whereas deep central cooling had no observable effect (Fig 4 left)

Since a moderate lowering of the temperature of the preoptic/anterior hypothalamic region was sufficient to elicit a full thyroidal response it seems justified to conclude that the thyroidal response to such cooling reflects a true physiological cold defence mechanism operating already at a moderate general hypothermia

From experiments of the present type it is not possible to say whether the thyroidal response is due to removal of a brake exerted by preoptic warmth detectors on the TSH releasing mechanism, or to stimulation of central hypothermia detectors or to a combination of both However a stimulatory inflow from peripheral cold receptors does not seem to be a prerequesite for the thyroidal response since this was obtained also when central cooling was performed with the animal in a hot environment

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From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

Fat in Twitch and Slow Muscle Fibres

By

JAN LANNERGREN

Received 9 October 1964

Cross striated muscle fibres have been divided into two groups depending on their appearance in the light microscope (a) fibres with Fibrillenstruktur in which the myofibrils are small of equal size and spaced in a regular lattice and (b) fibres with Felderstruktur in which the myofibrils are larger and irregular in size and spacing (e g Kruger 1952) On indirect evidence fibres with Fibrillenstruktur have been held for twitch fibres while those with Felderstruktur have been considered slow or "tonus fibres This view has been confirmed for frog muscle by light and electron microscopy on dissected fibres which had been identified as twitch or as slow fibres (Huxley and Peachey 1962) Ortmann (1951) found that Fibrillenstruktur fibres contained more fat than Felderstruktur fibres The hagfish slow fibres however contain more fat than the twitch fibres (Flood and Storm Mathisen 1962) Because of this discrepancy it seemed worth while to obtain direct evidence for the fat content of the twitch and the slow fibres in the frog

Bundles of 3—6 muscle fibres were dissected out in M ext dig long IV (Rana temporaria Rana ridibunda) leaving an intact nerve supply Selective stimulation of the low threshold nerve fibres to twitch muscle fibres was obtained at low current strength and of higher threshold fibres to slow muscle fibres at a critically increased current which blocked the larger fibres at an anode between the stimulating site and the muscle (Kuffler and Gerard 1947)

The localisations and physiological properties twitch or slow of each fibre in the preparation were noted The contractile mechanism was then inactivated by a solution of 95 mM K_2SO_4 before the preparation was fixed in a 4 % formaldehyde solution for 10 to 20 hrs The preparations were then checked microscopically and it was found that all the physiologically identified twitch fibres (10 fibres) were more or less opaque while all the identified slow ones remained clear (5 fibres) The preparations were then stained with the fat stain Sudan Black B according to Baker s method (Carleton and Drury 1957) The twitch fibres which were opaque after the fixing were now stained a distinct blue colour Under high power magnification the stain was seen to be

Fig 1 A bundle of 3 muscle fibres from M ext dig long IV stained with Sudan Black B One granulated twitch fibre centre and two clear slow fibres are seen

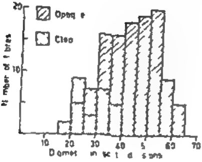

Fig 2 Histogram giving the distribution of opaque and clear fibres in 3 fixed unstained toe muscles Each column contains the fibres in diameter groups of 5 scale divisions on the ocular micrometer (1 scale division = 1 3 μ)

concentrated in ovoid granules oriented lengthwise in the fibre Fig 1) The slow fibres again which remained clear when fixed were almost unstained and without similar granulations Fig 2 gives the size distribution of opaque and clear muscle fibres in three toe muscles

This investigation was supported by a grant from Karolinska Institutet (Reservationsanslaget)

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Acta physiol scand 1965 63 195—196

From the Department of Chemistry Karolinska institutet King Gustaf V Research Institute and the Department of Internal Medicine Karolinska Hospital Stockholm Sweden

Inhibitory Action of Prostaglandin E_1 on the Mobilization of Free Fatty Acids and Glycerol from Human Adipose Tissue in Vitro

Prostaglandin and related factors

By

SUNE BERGSTROM and LARS A CARLSON

Prostaglandin E_1 (PGE_1) inhibits the catecholamine induced mobilization of free fatty acids (FFA) from rat adipose tissue incubated *in vitro* (Steinberg *et al* 1963 1964) and also *in vivo* in anesthetized dogs (Bergstrom Carlson and Oro 1964 Steinberg *et al* 1964). However we have recently observed that when PGE_1 was infused i v to man the concentration of FFA and glycerol in plasma increased (Bergstrom *et al* 1964 a and b). It was also observed that in man PGE_1 did not inhibit the noradrenaline induced mobilization of FFA and glycerol The difference between intact man and the studied animals with regard to the effect of PGE_1 on FFA might be due to different responsiveness of adipose tissue in various species To test this possibility we have studied the effect of PGE_1 on human adipose tissue *in vitro*

In each study subcutaneous adipose tissue was taken from a patient without known metabolic diseases during surgery in general anesthesia for uncomplicated gall bladder disease In all cases about 500 ml of a glucose fructose solution (5 per cent of each had been infused i v at the time of the biopsy The tissue was immediately placed in the incubation medium (room temperature) brought to the laboratory dissected free from connective tissue and cut into pieces weighing approximately 50 mg The pieces were randomly distributed into 24 incubation flasks each containing 7 ml of Krebs Ringer bicarbonate buffer pH 7 40 with ? per cent human serum albumin and 1 mg glucose/ml The flasks were weighed 2 ml taken off from each flask for analysis and various additions were then made Each flask was then immediately put into a water bath (37 C) and incubated with gentle shaking for 1 hr The time between the biopsy and the start of incubation has been less than 1 hr The procedures and analytical methods have been described earlier (Carlson 1963 Carlson and Östman 1963)

Data from ? typical studies are given in Table I and II PGE_1 significantly inhibited the basal release of glycerol and FFA from human adipose tissue There was no effect on glucose uptake

Noradrenaline stimulated the release of glycerol and PGE_1 significantly reduced this effect Since PGE_1 alone stongly inhibited the glycerol release it cannot be said whether it interfered with the hormonal activation of lipolysis in adipose tissue or acted by and independent mechanism.

The data unequivocally show that PGE_1 inhibits FFA mobilization from human adipose tissue as in other species The discrepancy between the effects of PGE_1 *in vivo*

Fig. 1. A bundle of 3 muscle fibres from M. ext. dig. long. IV stained with Sudan Black B. One granulated twitch fibre (centre) and two clear slow fibres are seen.

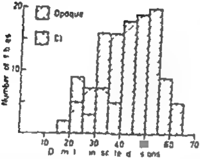

Fig. 2. Histogram giving the distribution of opaque and clear fibres in 3 fixed unstained toe muscles. Each column contains the fibres in diameter groups of 5 scale divisions on the ocular micrometer (1 scale division is 1.3 μ).

concentrated in ovoid granules oriented lengthwise in the fibre (Fig. 1). The slow fibres again, which remained clear when fixed, were almost unstained and without similar granulations. Fig. 2 gives the size distribution of opaque and clear muscle fibres in three toe muscles.

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Data from 2 typical studies are given in Table I and II. PGE_1 significantly inhibited the basal release of glycerol and FFA from human adipose tissue. There was no effect on glucose uptake.

Noradrenaline stimulated the release of glycerol and PGE_1 significantly reduced this effect. Since PGE_1 alone strongly inhibited the glycerol release it cannot be said whether it interfered with the hormonal activation of lipolysis in adipose tissue or acted by and independent mechanism.

The data unequivocally show that PGE_1 inhibits FFA mobilization from human adipose tissue as in other species. The discrepancy between the effects of PGE_1 *in vivo*

TABLE I Effect of noradrenaline and PGE_1 on the release of glycerol from human adipose tissue incubated in vitro The amount of adipose tissue in the incubation flasks varied between 270 and 420 mg The PGE_1 group contained 4 flasks all the other groups 5 flasks Mean value ± standard error of the mean P indicates the statistical significance of the difference from the control group (no addition)

Addition to the medium	None	Noradrenaline 0 1 μg/ml	Noradrenaline 1 μg/ml	Noradrenaline 10 μg/ml	PGE_1 1 μg/ml
Glycerol μmoles/g/h	0 47 ± 0 03	0 91 ± 0 09 P < 0 01	1 20 ± 0 16 P < 0 01	1 30 ± 0 04 P < 0 001	0 23 ± 0 07 P < 0 05

TABLE II Effect of noradrenaline and PGE_1 on the release of FFA and glycerol from and the uptake of glucose by human adipose tissue incubated in vitro The amount of adipose tissue in the incubation flasks varied between 500—700 mg Each group contained 6 flasks Mean value ± standard error of the mean P indicates the statistical significance of the difference from the control group (no addition)

Addition to the medium	None	Noradrenaline 1 μg/ml	PGE 1 μg/ml	Noradrenaline 1 μg/ml PGE 1 μg/ml
FFA μmoles/g/h	1 16 ± 0 04	1 50 ± 0 16 P < 0 05	0 69 ± 0 07 P < 0 001	1 12 ± 0 07 P 0 05
Glycerol μmoles/g/h	0 83 ± 0 05	1 45 ± 0 06 P < 0 001	0 25 ± 0 09 P < 0 001	0 91 ± 0 04 P 0 05
Glucose mg/g/h	0 80 ± 0 19	0 80 ± 0 11 P > 0 05	0 67 ± 0 11 P > 0 05	0 78 ± 0 17 P 0 05

and in vivo in man indicates a complex action of PGE_1 needing further studies to be elucidated

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Fusimotor Activity in the Spinal Cat

By

E. Alnæs, J. K. S. Jansen and T. Rudjord

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Abstract

Alnæs, E., J. K. S. Jansen and T. Rudjord. Fusimotor activity in the spinal cat. Acta physiol. scand. 1965. [illegible]. 197—212. — Fusimotor activity has been assessed from the changes in the response of muscle spindle receptors to a linear stretch and release of the muscle at various levels of fusimotor drive. The background fusimotor activity of the spinal cat caused an increased dynamic responsiveness of primary endings of ankle flexor as well as the ankle extensor muscles. Further dynamic activation of both flexor and extensor primary endings was observed during electrical stimulation of the ipsilateral saphenous and the contralateral lateral popliteal nerve. Definite signs of activity in static fusimotor neurones were not found in spinal preparations. The observations support the hypothesis of an independent control of the static and dynamic properties of primary endings of the muscle spindle. It is suggested that the activity in the dynamic fusimotor system largely is supported by dorsal root inflow, whereas the static fusimotor system mainly depends upon descending supraspinal activation.

A considerable body of evidence has been accumulated showing that fusimotor activity can have two fundamentally different effects on the response of muscle spindle receptors (For a review, see Matthews 1964). One is an increased dynamic responsiveness of the primary ending of the spindle. The other is an increased response to static extensions accompanied by a relative decrease in its response to dynamic stretch. Few attempts have been made to interpret fusimotor reflexes in terms of "dynamic" and "static" fusimotor systems (Jansen and Matthews 1961, 1962 a, Appelberg 196[illegible], 1963).

The object of the present investigation was to study the fusimotor activity in spinal animals from the point of view of the two different fusimotor systems. Fusimotor activity in the spinal preparation has been demonstrated by Hunt (1951) by recording the activity in the efferent fibres. With the present purpose of distinguishing between static and dynamic fusimotor effects it was necessary to record the afferent discharge from the spindle receptors (Granit and Kaada 1952) and to deduce the nature of the fusimotor activity from the changes in the response of the receptors.

13—633005.

The particular problems of the present paper are the nature of the background fusimotor activity in a flexor and an extensor muscle of the spinal cat and the changes in this activity during flexor and crossed extensor reflexes elicited by electrical stimulation of peripheral nerves.

Methods

Material. The results presented are based on experiments on 16 adult cats (2—4 kg). Eight of these were spinalized by section of the spinal cord at Th XII under Nembutal anesthesia (40 mg/kg administered i. p.). The final experiment on these cats was preformed on the following day. Eight cats were acutely spinalized under ether anaesthesia. All cats were acutely decerebrated intercollicularly by suction after ligation of the common carotid arteries. With the spinal cord cut, they all developed characteristic rigidity in the forelimbs.

Preparation. The left hind leg was completely denervated except for the nerve to the soleus or to the lateral gastrocnemius and the nerve to the anterior tibial muscle. The foot was disarticulated at the talo-crural joint and the anterior tibial and lateral gastrocnemius or soleus tendon isolated with a piece of bone from the insertions.

The spinal roots were exposed by lumbar laminectomy. In the first seven cats, the ipsilateral dorsal roots S I, L VII and L VI were cut initially, to eliminate possible autogenetic fusimotor reflexes from the extended muscle. Since it appears (Hunt 1951) that the background fusimotor activity of spinal preparations is largely maintained by dorsal root activity, these roots were left partly intact in the last 9 experiments. The dorsal roots were then sectioned only to the extent necessary for the isolation of spindle afferents. However, in these 2 groups of cats there was no apparent difference between the results, therefore the observations from both are presented together. The initial dissection and decerebration were completed under ether anaesthesia. The recording was done at least one hour after discontinuation of the ether administration in the unanaesthetized preparation. Exposed muscles and nerves were covered by liquid paraffin heated to body temperature and equilibrated with a gas mixture of 5 per cent CO_2 in oxygen.

The dorsal root L VII was split into fine filaments until only one active unit remained from one of the muscles studied. The unit was identified conventionally as a muscle spindle receptor by its pause during twitch contraction of the appropriate muscle (Matthews 1933). It was further classified as a primary or a secondary ending after determination of the conduction velocity of its afferent nerve fibre, 72 m/sec being accepted as the approximate dividing line between the two groups of receptors (Hunt 1954).

The peripheral nerves studied were cut peripherally and stimulated at a rate of 40/sec by means of indwelling electrodes. The stimulus intensities were from threshold for fusimotor effects to about 5 times the threshold for ipsilateral flexor reflexes.

Application of stretch. The cat was fixed in a frame and the left hind leg was imobilized with pins in the iliac crests and proximal and distal ends of the tibia. Through a steel hook in the tendon, the appropriate muscle could be connected to the piston of a cylinder (Meeman AB, Stockholm) (Fig. 1). By means of electromagnetic valves (Type EJV 3, Danfoss, Nordborg, Denmark) water at a pressure of about 5 kg/cm² could be directed to one or the other side of the piston which accordingly would move and stretch or release the muscle linearly. The cross section of the cylinder was 20 cm² and thus the available force was about 100 kg. Compared to this, the opposing forces from the muscles were small and would not noticeably affect the rate of extension. In the present experiment the velocity of stretch was kept at its maximum with the available water flow. This was about [illegible] mm/sec and remained constant in any one experiment. Because of variations in water pressure, there were slight differences in the speed of extension in different experiments, but the speed always remained between 17 and 19 mm/sec.

Usually, the muscles were stretched 15 mm at which point the length closely corresponded to the greatest physiological length of the muscle. Because of the large passive tension developed by the gastrocnemius at full physiological extension, this muscle was stretched to 3 or 4 mm short of this length. The stroke of the stretcher was interrupted automatically by micro-switches at the end of the extension or release, and the muscle then remained in the terminal position. With an additional switch, the stroke could be interrupted at any position during the movement (Fig. 1).

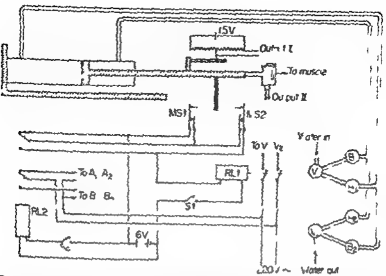

Fig 1 Diagram of hydraulic pump used to stretch the muscle and transducers for length and tension recording. Piston movement is controlled by six electromagnetic valves (V, V2, A, A2, B1, B2) operated indirectly through relays RL1 and RL2. The movement is stopped in end positions by mechanically operated microswitches (MS1, MS2). A manual switch (S1) is used for stopping and starting the piston in any intermediate position. The direction of movement is determined by the position of switch S1. At the output I a length signal is obtained from a linear wire wound potentiometer. The stretch is applied to the muscle through an isometric myograph. Output II is the output of the mechano-electronic transducer valve of the myograph.

Recording. The action potentials of the dorsal root filaments were fed differentially through a Tektronix 122 preamplifier and displayed on the stationary spot of a Tektronix 502 oscilloscope. Usually the activity of two filaments were recorded simultaneously on the two available channels. On a second Tektronix 502 oscilloscope one beam was used to record the muscle length. This signal was obtained from a wire wound linear potentiometer connected to the piston shaft of the stretcher (Fig. 1). The tension signal displayed on the second beam was supplied from an isometric myograph with a RCA 5734 valve as transducing element (Fig. 1). On the same beam time marks from a Tektronix 180A pulse generator were displayed as well. The second oscilloscope was mounted at 90 degrees to the first and its signals photographed through the accessory lens and the mirror of a Grass C4 camera. A film speed of 10 cm/sec was used throughout the experiments. The discharge frequency of the receptors was measured on the recording paper by counting the number of spikes occurring in a 0.1 sec period. The accuracy of the measurement was better than ? per cent.

Experimental procedure. In ... all ... number of spindle receptor afferents from the ... flexor and extensor muscle studied were ... and identified. The endings of one muscle were then studied usually two at a time during the standard linear stretch of the muscle. For each pair the effect of peripheral nerve stimulation on the ipsilateral saphenous nerve and the contralateral lateral popliteal nerve was also determined. Then the second muscle and its endings were studied in the same way. Having thus obtained the response of all the endings to the standard stretch and to nerve stimulation the ventral roots L VI, L VII and S I were cut. About 10 min later because of possible injury discharges in the ... ventral ... the response of the de-efferented endings to the same extension of the muscle was determined. The period of recording was usually completed in ? hrs. Post mortem examination confirmed the completeness of the ventral root and spinal cord section.

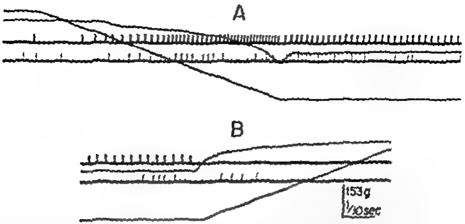

Fig. 2. A Response of primary ending (above) and secondary ending (below) to standard linear stretch of the de-efferented anterior tibial muscle. Additional signals show muscle length (above initially) and muscle tension. B Response of same endings during release of extension. Total extent of stretch 15 mm terminating at full physiological extension of the muscles

Results

As the effects of the fusimotor activity must be assessed in relation to the behaviour of the receptor without efferent innervation the properties of the de-efferented receptors will be dealt with initially. These passive properties of soleus spindle receptors have been described by Matthews and collaborators (Harvey and Matthews 1961 Jansen and Matthews 1962 a b Matthews 1963). However the muscle spindle receptors of the anterior tibial muscle and the lateral gastrocnemius have not previously been systematically studied

A *Passive properties of anterior tibial spindle receptors* From the point of view of functional characterization two parameters of spindle receptor behaviour appear to be of particular importance namely static sensitivity which is a measure of the sensitivity of the receptor to changes in length of the muscle and dynamic sensitivity which is a measure of the sensitivity to the rate of change of muscle length. The static sensitivity of the receptor can be determined by measuring its discharge frequency at various degrees of static extensions. A measure of dynamic sensitivity can be obtained from the so called dynamic response of the receptor (Jansen and Matthews 1962 a) which is the difference between the firing frequency at the end of a dynamic stretch and that after 0.5 sec of maintained extension (cf Matthews 1963). In the following the term dynamic index will be adopted for the dynamic response as suggested by Crowe and Matthews (1964 a)

Fig 2 shows a characteristic example of the behaviour of a primary and a secondary ending during our standard linear stretch and release of the anterior tibial muscle. At the same time it provides an example of the type of record on which most of the measurements of the present study have been made. It is clearly seen that the discharge frequency of both types of receptors increased during the extension of the muscle. In addition the primary ending displayed a marked reduction in the frequency of firing at the transition from dynamic to static extension. Characteristically the frequency of

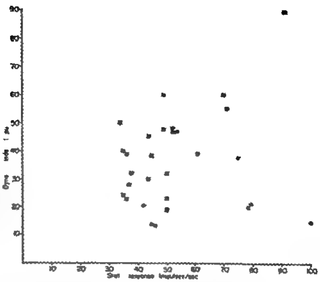

Fig 3 Scatterdiagram of dynamic index (ordinate) and static response (abscissa) of deefferented anterior tibial primary (■) and secondary endings (●) in response to standard linear stretch (18 mm/sec) of the muscle

firing of the secondary ending is not markedly different from that of the primary but the dynamic index of the secondary ending is smaller Another typical feature of primary and secondary behaviour appears at the release of the muscle (Fig 2B) The primary ending stopped firing immediately on release whereas the secondary ending continued firing with decreasing frequency during release Of the 42 primary endings comprising the present study only 2 fired during the release of extension at the rate commonly employed (18 mm/sec) Of the 17 secondary endings on the other hand 8 fired two or more impulses during releases at the same rate This difference in dynamic index and behaviour during release of the muscle agrees well with the observations of Harvey and Matthews (1961) on primary and secondary endings of the soleus muscle In their material a greater fraction of the primary and secondary endings discharged during the release of stretch but this is probably a consequence of the smaller rate of release (3 mm/sec) employed by Harvey and Matthews

In the scatter diagram of Fig 3 the dynamic index and static response of the entire present series of anterior tibial muscle spindle receptors are illustrated Each point represents the observations on one receptor during and after a standard linear stretch of the muscle up to full physiological extension The static response was measured as the frequency of firing 0 5 sec after the end of the dynamic stretch In spite of an appreciable overlap in the size of the dynamic indices of the two groups of receptors it appears from Fig 3 that the dynamic index of the secondary endings on the whole is smaller than that of the primary endings Statistically the difference between the mean dynamic index of the primary (45 imp sec) and the secondary endings (23 imp sec) is highly significant (t test P 0 1 per cent Furthermore it should be noted that the two secondary endings with the largest dynamic indices had fairly large static responses as well If therefore the criteria of percentage slowing of Harvey and Matthews (1961) had been employed the difference between the two groups of endings would have been greater

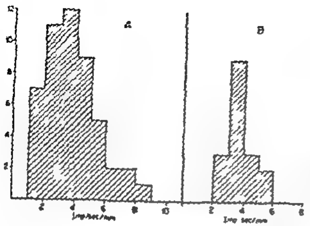

Fig. 4. Histograms of static extension slopes of de-efferented anterior tibial primary endings (A) and secondary endings (B).

Another feature of possible importance can be seen in Fig. 3. The mean static response of the secondary endings (62 imp/sec) is higher than that of the primary endings (47 imp/sec). This may be due to a greater average static sensitivity of the secondary endings or a lower threshold of these endings. This question will be dealt with later.

The observations of Fig. 3 can be compared with the corresponding observations of soleus spindle receptors reported by Matthews (1963). From his Fig. 10 it may be seen that soleus primary endings at 18 mm/sec stretch velocity had dynamic indices of 50 to 135 imp/sec and the secondary endings had dynamic indices of about 10 to 35 imp/sec. Thus it appears that the dynamic index of the primary endings of the anterior tibial muscle is in the lower range and below the comparable value of soleus primary endings. The dynamic index of the secondary endings, on the other hand, is in approximately the same range as that of the soleus secondary endings. Therefore, there is apparently a greater overlap in dynamic sensitivities of primary and secondary endings in the anterior tibial muscle than in the soleus. However, it should be pointed out that the values of Matthews (1963) were obtained after exclusion of all units with conduction velocity between 60 and 80 m/sec. In addition, 10 per cent of the other units did not fit into the general picture presented in his Fig. 10. In Fig. 3, on the other hand, all the units of the present material have been included. Of these, only 5 had conduction velocities of the afferent fibre between 60 and 80 m/sec, and these units did not appear to contribute particularly to the overlap observed. Therefore, the conclusion is probably valid that there is a greater degree of overlap in dynamic sensitivities of anterior tibial primary and secondary endings than in the soleus spindle receptors. This greater overlap is mainly due to a smaller average dynamic sensitivity of the primary endings in the anterior tibial muscle. Bianconi and van der Meulen (1963) found some overlap in dynamic responsiveness of primary and secondary endings. They tentatively ascribe dynamically sensitive secondary endings to receptor terminals situated in the myotube region. Their results are, however, not directly comparable with the present observations.

The static frequency of firing of muscle spindle receptors increases approximately linearly with increasing static extension (Eldred, Granit and Merton 1953; Jansen and Matthews 1962 b). The slope of this frequency-extension relation has been defined as the static sensitivity of the ending (Whitteridge 1959). Such frequency-extension curves have been obtained for the present anterior tibial spindle receptors during interrupted extensions up to a final length closely corresponding to full physiological extension of the muscle. Straight lines have been fitted by eye to the observations and the values of the slopes of these lines are presented in Fig. 4. The static sensitivity was determined in the same manner as that employed by Jansen and Matthews (1962 b).

A

B

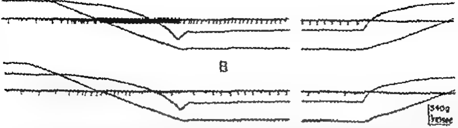

Fig. 5. Effect of ventral root section on response of soleus primary ending to standard stretch and release of the muscle. A: Ventral roots intact. B: After section of ipsilateral ventral roots L VI, L VII and S I. Length signal above and tension signal in middle initially.

and the values should accordingly be directly comparable to the values of soleus spindle receptors given by these authors. The mean value of the static slope of the primary endings of Fig. 4 was 3.7 imp/sec/mm (S. D. 1.6). That of the secondary endings was 3.6 imp/sec/mm (S. D. 0.9). There is no significant difference between these two values, and furthermore there is no significant difference between the present values and those of the soleus receptors given by Jansen and Matthews (1962 b). Thus one can conclude that the static sensitivities of primary and secondary endings in soleus as well as in the anterior tibial muscles are all very similar. In the rabbit, however, Granit and Homma (1959) found some spindle receptors in the anterior tibial muscle with static sensitivities of more than 20 imp/sec/mm. At present no explanation can be given for this difference between the two species.

The static sensitivity of anterior tibial muscle spindle receptors has also been investigated by Fehr (1962). He found in a limited material a higher static sensitivity for the primary endings than for the secondary endings. His values were entirely within the range of those of the present report and the difference between the 2 types of receptors is not supported by the present material. Fehr (1962) also found that a greater proportion of the secondary endings showed a "spontaneous" firing at the initial length, whereas the majority of the primary endings were silent in the unloaded muscle. This is partly supported by the present material. In the unloaded muscle, 23 of the 42 primary endings had a resting discharge, and the same applied to 12 of the 17 secondary endings. The mean frequency of the primary endings in the unstretched muscle was 7 imp/sec. That of the secondary endings was 15 imp/sec. This explains the greater average static response of the secondary endings compared to that of the primary endings of Fig. 3.

The greater frequency of spontaneous discharge in secondary than in primary endings of the anterior tibial muscle is of some interest. Presumably it indicates a slightly lower threshold for the anterior tibial secondary endings. In the soleus and gastrocnemius of the cat the relationship is the opposite with a higher incidence of spontaneous discharge and a lower threshold in the primary endings (Hunt 1954, Harvey and Matthews 1961). In the gastrocnemius and plantaris muscle of the rabbit, on the other hand, Diete-Spiff (1961) found spontaneous firing more frequently among the secondary endings.

B. *Spontaneous fusimotor activity.* Other factors being constant, a change in the response of a muscle spindle receptor to a standard stretch after interruption of the fusimotor nerve fibers must be ascribed to the elimination of activity in its fusimotor supply. On such evidence Jansen and Matthews (1962 a) showed that the response of the great majority of spindle receptors in the soleus of the decerebrate cat is appreciably increased by the background fusimotor activity in this preparation. They found, further

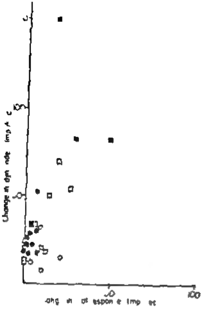

Fig 6 Scatter diagram of effects of ventral root section on dynamic index (ordinate) and static response (abscissa) of anterior tibial (circles) and lateral gastrocnemius or soleus primary end ngs (squares) Open symbols observations from acutely spinalized cats Filled symbols from cats spinalized the previous day

more that for the different primary endings there was no simple relationship between the degree of change in dynamic and static responsiveness caused by this fusimotor activity

In the spinal cat the change in respon e of a muscle spindle receptor after section of the appropriate ventral roots was usually not as marked as that of soleus spindle receptors in the decerebrate cat This applies to flexor as well as extensor spindles examined in the present study Of the 88 primary endings of the anterior tibial muscle and lateral gastrocnemius and soleus about 60 per cent did not show any appreciable difference in response after deefferentation The change in response that was found in 33 of these primary endings however was quite characteristic and les complicated than that described for primary endings in soleus of the decerebrate cat (Jansen and Matthews 1962 a) Fig 5 provides an example of a fairly marked but typical effect The response of a primary ending in soleus with intact ventral root supply is shown in A It is clearly seen that the receptor was very sensitive to the dynamic phase of the stretch and it was immediately silenced at the release of the extension In B the respon e of the same receptor is shown after section of the ventral roots L VII and S I Then there was a large reduction in its frequency of firing during the dynamic stretch On the other hand the firing frequency under static conditions at the initial and final length was only moderately reduced Similar but usually less pronounced changes have also been observed for primary endings in the anterior tibial and lateral gastrocnemius muscles

Originally the experiment were performed on acutely spinalized cats As clear cut changes in response were only found in about one third of the primary endings the possibility of a fairly longlasting depression of fusimotor activity after spinalization was con idered Therefore similar experiments were done on cats spinalized on day before the final experiment The most important observations of these series of experiments are presented in Fig 6 As mentioned above the total material consi ted of 88

primary endings of which 42 belonged to the anterior tibial muscle and 46 to the lateral gastrocnemius and soleus Approximately equal numbers of these two groups were from the acutely and the chronically spinalized cats Arbitrarily a change of less than 10 imp/sec in either dynamic or static response of a receptor after section of the ventral roots was considered not significant. Such small changes are probably within the range of change that could be ascribed to unspecific causes as the two measurements usually were performed with an hour or greater intervals Therefore the 55 units with this small degree of change in response have not been included in the graph of Fig 6 In this scatter diagram each point represents the observations on one ending The change in dynamic index after ventral root section has been plotted along the ordinate and corresponding change in static response along the abcissa It appears from the diagram that none of the units exhibited large changes in static responses and furthermore that all of them had a moderate or large decrease in dynamic index after section of the ventral roots This should be compared with similar observations on extensor spindles in decerebrate cats (Jansen and Matthews 1962a Jansen and Rudjord 1965) The spindle receptors described by these authors show increases in the static response and either decreases or increases in dynamic index on account of the fusimotor activity prevailing in the decerebrate cat

The changes in response plotted in Fig 6 apply to anterior tibial as well as to gastrocnemius-soleus spindle receptors and to acutely spinalized cats as well as to cats spinalized one day before the final experiment There appear to be some differences among these various groups such as a smaller degree of change for anterior tibial than for gastrocnemius soleus primary endings but it should be remembered that the material of Fig 6 is selected on the basis of presence of fusimotor effects Furthermore considering the variation from cat to cat and even among the primary endings of a given muscle in one cat the important observation of Fig 6 is that this kind of change in response of primary ending after ventral root section is fairly common and occurs in flexor as well as extensor primary endings Also other kinds of change in response ascribable to background fusimotor activity have not been observed in spinal cats The possible reality of small differences between the various groups of Fig 6 must be left for more extensive investigations

Above attention was drawn to another important feature of Fig 5 namely the cessation of firing during release This is a typical finding for primary endings without fusimotor support In the decerebrate cat on the other hand firing during release is a very frequent consequence of fusimotor activity (Jansen and Rudjord 1965) In the material of Fig 6 firing during release was never observed although many of the endings were quite heavily influenced by fusimotor activity as for instance that of Fig 5

Yet another difference exists between the observations in the present series of experiments on spinal cats and those on decerebrate cats (Jansen and Matthews 1962 a In the decerebrate cat the static response of the majority of the secondary endings in the soleus is increased on account of background fusimotor activity and the increase in static firing frequency of the secondary endings is of the same order of magnitude as that of the primary endings In the present material on spinal cats the behaviour of 17 secondary endings of the anterior tibial muscle and 5 secondary endings of soleus and gastrocnemius have been determined with and without intact ventral root supply Of these 22 secondary endings only one showed a decrease in response of more than 10 imp sec to a stretch up to full extension of the muscle The one exception belonged to the lateral gastrocnemius and showed a decrease in response of 20 imp sec which

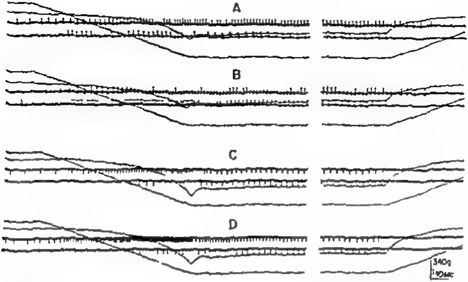

Fig. 7. Effect of stimulation of spinal nerves. A. Control response of anterior tibial secondary (above) and primary ending (below). B. Response of same two endings during repetitive stimulation of the ipsilateral saphenous nerve at 40 impulses/sec. Intensity just above threshold for flexion reflex. Length (above) and tension (below) signals 50 msec misaligned in A and B. C. Control response of soleus primary ending (above) and secondary ending (below). D. Response of same endings as in C during repetitive stimulation of the contralateral lateral popliteal nerve at 40 impulses/sec. Intensity of stimulation above threshold for ipsilateral flexion reflex, below threshold for contralateral extension reflex.

was about 20 *per cent* of its total response to the final length. Thus the conclusion was reached that the secondary endings are not, or only very moderately, influenced by the background fusimotor activity in the spinal cat.

C. *Reflex activation of fusimotor systems.* It is well known that stimulation of various spinal nerves can induce fusimotor activity in spinal preparation (Hunt 1951, Hunt and Paintal 1958, Voorhoeve and van Kanten 1962). In order to be of value from the present point of view, namely the degree of differential activation of the static and the dynamic fusimotor apparatus, such reflex activation should preferably be maintained and stable during the period needed to perform the standard stretch. This is definitely not so, and it introduces a limitation on the conclusions that can be drawn from the observations to be presented below. On the other hand, the effect of such nerve stimulation was surprisingly uniform, which suggests that it represents a typical pattern of fusimotor activation.

Two nerves were selected for stimulation, the contralateral lateral popliteal nerve and the ipsilateral saphenous nerve. The former was chosen to introduce a reflex activation in the crossed extensor pattern, the latter, a flexor reflex pattern. The nerves were always stimulated repetitively at a frequency of 40/sec for about one sec before and throughout the test stretch. It was attempted to obtain fusimotor activation without simultaneous extrafusal activation of the muscle studied, but stronger shock intensities were also employed. In general the intensities used were from about 0.8 times the thre

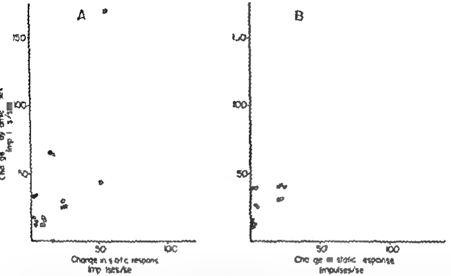

Fig 8 Scatter diagram of effects of stimulation of ipsilateral saphenous nerve (A) and contralateral lateral popliteal nerve (B) on response of anterior tibial (circles) and lateral gastrocnemius and soleus (squares) primary endings Changes in dynamic index plotted along ordinate and changes in static response along abscissa Open symbols observations from acutely spinalized cats filled symbols from cats spinalized the previous day

shold for ipsilateral flexor reflexes up to about 5 times this value So far no attempt has been made to determine which particular group or groups of nerve fibres in the stimulated nerves were responsible for the reflex effects

Typical examples of the effects of nerve stimulation are given in Fig 7 The first 2 records show the response of a primary and secondary ending of the anterior tibial muscle to a standard stretch A is the control response and the records of B were obtained immediately afterwards during stimulation of the ipsilateral saphenous nerve The stimulus intensity was just above threshold for the ipsilateral flexor reflex The greater part of the reflex of the muscle had subsided at the time of the test stretch about one sec after the beginning of stimulation The residual of the activity can be seen as slight irregularities in the tension record in B There was no increase but actually a slight decrease in the firing frequency of the secondary ending compared to that of the control stretch The firing frequency of the primary ending on the other hand was markedly increased during the dynamic phase of the stretch Its firing frequency during the maintained stretch at full extension was increased by only 20 imp sec however and it stopped discharging immediately at the release

A very similar pair of records is shown in Fig 7 C and D These illustrate the responses of a primary and a secondary ending of the soleus C is the control stretch and D was obtained shortly afterwards during stimulation of the contralateral lateral popliteal nerve The stimulus intensity was just above threshold for the ipsilateral flexor reflex but below that of crossed extensor effects Again it is seen that the response of the secondary ending was hardly influenced by the nerve stimulation There was a

marked increase in the dynamic frequency of firing of the primary ending whereas its discharge during static stretch was only moderately elevated and it was immediately silenced during the release

The kind of changes in spindle receptor responses illustrated in Fig 7 was characteristic of the present material and very few exceptions were found A summary of the observations on primary endings is given in the scatter diagrams of Fig 8 Often appreciable amounts of simultaneous extrafusal contractions were observed during nerve stimulation However it is not thought that these extrafusal effects were responsible for the changes in the response of the receptors because the typical increase in dynamic responsiveness of the primary endings was frequently observed without noticeable activity and furthermore there is no obvious reason why extrafusal contractions per se consistently should increase the dynamic responsiveness of the primary endings

The graphs of Fig 8 have been produced by comparing the response of a primary ending during a control stretch and during continuous nerve stimulation shortly afterwards Each point represents the observations on one ending The change in dynamic index has been plotted along the ordinate and the change in static response along the abscissa The observations during ipsilateral saphenous nerve stimulation are presented in A and those during contralateral lateral popliteal nerve stimulation in B As before all the observations exhibiting less than 10 imp/sec change in either static or dynamic response have been excluded from the graphs In addition the observations on 6 units have been excluded on account of large and irregular extrafusal contractions None of these showed any definite signs of fusimotor activation and 4 of them showed small to moderate decreases in static response as well as dynamic index during nerve stimulation

Altogether 82 primary endings have been examined during stimulation of the ipsilateral saphenous nerve 45 of these observations are presented in Fig 8 A It appears that the changes observed were always a moderate to large increase in dynamic index In general the change in static response was much less pronounced This applies to primary endings of the anterior tibial muscle as well as gastrocnemius and soleus There was thus no apparent difference between the responses of flexor and extensor spindles to saphenous nerve stimulation There was furthermore no definite difference between the observations on acute spinal preparations and animals spinalized one day earlier

During stimulation of the contralateral lateral popliteal nerve a total of 83 primary endings have been studied Of these the observations made on 28 appear in Fig 8 B Again the graph shows that there was a definite and sometimes quite large increase in the dynamic index of these units with only small or moderate changes in the static response This applies to both anterior tibial and to soleus gastrocnemius receptors The response occurred more frequently in cats spinalized the day before the final experiment but it was found in the flexor as well as the extensor muscles also in acutely spinalized cats In connection with Fig 8 A B one point deserves special mentioning The graphs show the change in response beyond that of the control stretch For some endings dynamic index was appreciably increased already in the control stretch on account of the background fusimotor activity (cf Fig 6) In some instances the nerve stimulation would cause only a slight further increase in the dynamic index of such endings and it would therefore appear as only a slight change in the graphs of Fig 8

A further point concerning the nerve stimulations is their effect on the discharge during release of the stretch Such firing consisted only of 1 or 2 impulses and was

observed in only 6 of the 76 primary endings observed during stimulation of the ipsilateral saphenous and contralateral lateral popliteal nerve This is in marked contrast to the effect of similar nerve stimulation in the decerebrate cat (Jansen and Rudjord 1965)

Observations have been made on the behaviour of a limited number of secondary endings during stimulation of the two spinal nerves 11 of these belonged to the anterior tibial muscle and had conduction velocities of their afferent fibres between 27 and 62 m/sec None of these endings showed a change in response of more than 5 imp/sec during stimulation of either the ipsilateral saphenus or the contralateral lateral popliteal nerves 8 of these units were studied in acutely spinalized cats Two units with afferent fibre conduction velocities of 66 and 67 m/sec showed a moderate increase in response (about 15 imp sec) during saphenous nerve stimulation Otherwise these two endings behaved like secondary endings with small dynamic index and firing during release after section of the ventral roots

The observations on secondary endings in gastrocnemius soleus are even more limited Only 5 secondary endings in these muscles have been observed during nerve stimulation The conduction velocity of their afferent fibres was from 42—62 m/sec One of these belonging to the lateral gastrocnemius showed a definite increase in firing rate during stimulation of the ipsilateral saphenous nerve and a less pronounced increase in response during contralateral lateral popliteal stimulation The other four endings did not exhibit convincing changes in response during nerve stimulation

It has been pointed out before (Hunt and Paintal 1958 Jansen and Matthews 1962 a) that stimulation of peripheral nerves may have fairly longlasting effects on the fusimotor activity even after the cessation of the stimulus This has been observed in the present experiments as well In some spinal cats the phenomenon has been very pronounced The dynamic activation of the primary endings by nerve stimulation could sometimes last for two to three minutes after stimulation As judged from the response of a primary ending the fusimotor activity was declining slowly during this period

Discussion

The main finding of the present work is that the background fusimotor activity of the spinal cat cause a marked increase in the dynamic frequency of firing of the spindle primary endings with only a small increase in their response to static extensions In general this may correspond to the common observation of a lack of tonic stretch reflexes in spinal cats Their responses to dynamic stretches like tendon taps on the other hand are quite brisk

The present observations can be interpreted in terms of two fusimotor systems It was shown (Figs 5 6) that in the spinal preparation there was a dominance of activity in a fusimotor system mainly causing increased spindle response during dynamic extensions It appears entirely reasonable to identify this system with the efferent fibres inducing comparable effects demonstrated by Matthews 1962 Whether there is at the same time some background activity also in the static fusimotor system in spinal cats is impossible to decide from the present experiments One observation definitely speaks against the possibility of appreciable static fusimotor activity in spinal cats That is the complete absence of firing during the release of extension in these preparations In the

subsequent paper (Jansen and Rudjord 1965) it is shown that even a small amount of static fusimotor activity can induce firing during release of comparable extensions

In the experiments with stimulation of single efferent fusimotor fibres it is difficult to establish definitely the relationship between the static and dynamic effects and Boyd's γ_1 and γ_2 fibres (Matthews 1962 Crowe and Matthews 1964 a b) In accordance with the suggestions of Jansen and Matthews (1962 a), it is argued that the dynamic fusimotor fibres are the γ_1 fibres of Boyd innervating the nuclear bag intrafusal fibres and that the static effects are induced by stimulation of the γ_2 fibres innervating the nuclear chain intrafusal fibres The present observations lend some support to these inferences It is known that the receptor terminals of the secondary ending are mainly located on the nuclear chain intrafusal fibres (Boyd 1962) The lack of effect of ventral root section on the response of the secondary endings suggests that the dynamic fusimotor effects observed in spinal cats were due to activation of the nuclear bag intrafusal fibres

Hunt (1951) found by recording the activity of γ efferent fibres in the ventral roots of spinal cats that the background activity in these fibres was completely abolished after bilateral section of the dorsal roots Presumably, the efferent activity recorded by Hunt is responsible for the increased dynamic responsiveness of the primary endings in spinal animals demonstrated in the present investigation This dynamic fusimotor activity is accordingly largely supported by dorsal root inflow On the other hand Eldred *et al* (1953) emphasize the lack of effect of extensive dorsal root section on background fusimotor activity in the decerebrate cats However the latter authors were mainly studying the afferent discharge from spindle receptors under static extensions of the muscle and would therefore not notice a possible change in the dynamic fusimotor system of their preparation

The absence of static fusimotor reflexes during nerve stimulation in the spinal preparation requires an explanation No direct evidence is available on this point It may be related to the observations of Granit Holmgren and Merton (1955) of a lack of fusimotor coactivation in various reflexes after a lesion of the cerebellum Granit *et al* (1955) were studying fusimotor effects under static conditions and it is conceivable that the spinal reflex pathways for static fusimotor activity largely depend upon descending facilitatory inflow possibly from the cerebellum, in order to be available for activation

A final point is relevant in this connection Hunt (1951) and Hunt and Paintal (1958) found that of the fusimotor fibres to a muscle in the spinal cat only about 30 per cent showed a background discharge and furthermore that a number of the fusimotor fibres studied were not reflexly activated from any of the nerves they investigated Such unresponsive units usually exhibited no background discharge This finding may be correlated with the present observation of a complete lack of static fusimotor effects in the spinal cat

In conclusion it may be suggested that the activity in the dynamic fusimotor system to a large extent is supported by purely spinal mechanisms driven by dorsal root afferent activity Whether this is the main source of dynamic fusimotor activity or whether suprasegmental structures contribute appreciably to this remains to be elucidated The activity in the static fusimotor system on the other hand appears to depend entirely on facilitatory systems descending from the brain

Considering finally the possible functional significance of the dynamic fusimotor activity in the spinal cat it should be noticed that this activity was readily induced by

the nerve stimulations with surprisingly little specifity in the present type of experiments. Flexor as well as extensor spindles were excited by the ipsilateral as well as the contralateral nerve and the effects of nerve stimulations often outlasted the stimulus for one minute or more. If we now consider the muscle as placed in a control loop in the servo theory sense with the output of the primary endings of the spindles as the negative feed back signal then as pointed out by Matthews (1964) the effect of increasing the dynamic sensitivity of the receptors will be a general damping of movements in the system. In these terms the motor apparatus of the spinal cat is a damped system and the damping is readily increased by non specific spinal inputs. The damping effect will make itself felt in two different ways: firstly in the simple control loop in which each muscle is placed and secondly by the Ia reciprocal inhibition from the antagonist muscle which of course under physiological conditions always moves with the agonist but in the opposite direction. If one regards the spinal motor apparatus as a kind of basic mechanism on which the higher motor centers of the brain exert their influence it is of interest from the point of view of the stability of the system that appreciable damping appears to be an important element in this basic motor outfit.

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The Secretion of Adrenal Medullary Hormones During Hypoglycemia in Intact, Decerebrate and Spinal Sheep[1]

By

CHRISTIAN CRONE[2]

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Abstract

Crone, C. *The secretion of adrenal medullary hormones during hypoglycemia in intact, decerebrate and spinal sheep.* Acta physiol. scand. 1965. 63. 213—224. — It is not known from which part of the central nervous system the adrenal medulla is activated in hypoglycemia. The question was investigated in animals with various parts of the central nervous system removed, and the adrenal response was compared with that of intact animals. The amounts of adrenaline and noradrenaline in adrenal effluent blood were determined fluorimetrically. Intact animals showed increased secretion rate of adrenaline and to a lesser degree of noradrenaline in response to hypoglycemia. Decerebrate animals were capable of activating their adrenal medulla during hypoglycemia while spinal animals did not increase the rate of secretion above the resting level. It is therefore concluded that sympathetic neurones situated in the brain stem caudal to the hypothalamus are capable of activating the adrenal medulla in hypoglycemia. It proved very difficult to lower the blood glucose concentration in decerebrate animals despite the administration of large doses of insulin. The mechanism behind the insulin resistance of the decerebrate animal is discussed.

Secretion from the adrenal medulla is due to activity in the central nervous system, the denervated adrenal medulla being almost insensitive to stimuli which are known to increase the secretion rate of catecholamines (Comline and Silver 1961). It is not known whether all parts of the central nervous system possess the ability to stimulate the adrenal medulla, or whether discrete regions are responsible. Dunér (1953) found some evidence that a region in the hypothalamic area might control the rate of secretion from the adrenal medulla when the blood sugar was elevated above normal values. Whether the activation which takes place in hypoglycemia is due to hypothalamic ac-

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[1] The results reported in this article were in part communicated at the XI Scandinavian Physiological Congress in Copenhagen (Crone 1963).

tivity is however unknown and the aim of the present work has been to clarify this problem by comparing the adrenal response to hypoglycemia in intact animals and in animals with different parts of the brain removed.

The relative amounts of adrenaline and of noradrenaline in the adrenal effluent may vary under different circumstances (Euler 1956) Dunér (1954) found that in the cat hypoglycemia leads to a preferential secretion of adrenaline In the present work I have investigated whether this also occurs in the sheep

An analysis of the adrenal medullary response in hypoglycemic sheep has recently been published by Setchell and Waites (1962) who however used unspecific methods for evaluating the amounts of catecholamines in plasma and they did not differentiate between adrenaline and noradrenaline

Methods

The techniques used were adopted from those described by Comline and Silver (1961) and that paper should be consulted for further details

Thirtytwo expts were performed on Welsh mountain sheep and lambs Eight expts were discarded because the blood pressure fell too much and ? because of bleeding from the brain stem The animals were between 9 and 18 months old

Anesthesia was established by i. v. injection of sodium pentobarbitone (Nembutal) Initially about 30 mg kg was injected and small supplementary doses were injected as needed In some experiments chloralose (80 mg kg i v) was used but as the blood pressure tended to be lower in these experiments Nembutal was preferred and only results from those experiments are reported here

Effluent blood was collected from the left adrenal gland The method of preparation cannulation and collection of the adrenal effluent blood was similar to that described by Silver 19?9) Between the samplings the blood was returned through a Murphy drip chamber to the external jugular vein This vein was preferred to the femoral vein as its larger diameter permitted insertion of bigger cannulae so that the resistance could be kept as low as possible All animals were tracheotomized and in experiments on spinal animals artificial respiration was given In the majority of the experiments Dextran (Pharmacia Ltd) was given prophylactically before operating on the adrenals Experiments in which a lower blood pressure than 70 Hg occurred were rejected except those performed on spinal animals where the blood pressure always fell to about 30 mm Hg after transection of the cord

Before establishing the external circuit for collection of adrenal blood Heparin was given i v (10 mg/kg over a period of 4—5 hrs)

Hypoglycemia was induced by i v injection of insulin (Burroughs Wellcome and Co) In experiments on intact sheep 2 IU kg were given The dose was progressively increased to 10 IU/kg) in experiments on decerebrate sheep because these proved to be very resistant to insulin

Decerebration The vault of the skull was cautiously removed with bone nibblers The brain stem was cut immediately above the superior colliculi Firm application of cotton wool to the cut circle of Willis prevented bleeding At least 1 1/2 hrs elapsed between the completion of the decerebration and the injection of heparin

Transection of the spinal cord This operation was performed immediately below the foramen magnum through a laminectomy of the first second and third vertebrae The brain was destroyed through the foramen magnum and the circulation to the entire head was cut off by a firm ligature around the neck.

Determination of adrenaline and noradrenaline The hormones were estimated fluorimetrically on plasma eluates according to the technique described by Lund (1949) and modified by Price and Price (1957) and by Euler and Lishajko (1959) The adjustments to different pH during the various steps of the analysis were carried out by use of a pH meter which permitted continuous control of the pH The lutines formed by oxidation with ferricyanide were stabilized with ethylenediamine A plasma sample taken from an unanesthetized animal underwent the same treatment as the samples from the experiment and served as blank It soon became

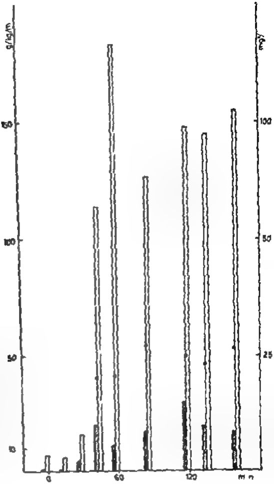

Fig 1 The output of adrenaline and noradrenaline from the left adrenal gland of a sheep during development of hypoglycemia Abscissae Time in minutes Zero-time Injection of insulin 2 IU/kg Ordinates Output of catecholamines in ng/kg/min, Adrenaline ☐ Noradrenaline ▣ Concentration of glucose in blood ●——●

evident that the recovery of catecholamines from the plasma could vary from analysis to analysis and a sample containing known amounts (1 μg) of the two hormones was therefore analyzed together with the unknown samples This precaution made it possible to correct for losses The average recovery of adrenaline was 70 5 (coefficient of variation 10 3 $n = 35$) and of noradrenaline 66 █ (coefficient of variation 11 3 $n = 33$ The mean correction factors thus were 1 42 for adrenaline and 1 50 for noradrenaline somewhat higher than the value indicated by Lund 1 25 (1949) The amount of adrenaline and noradrenaline in all samples are expressed as the base and not as the salt

Determination of adrenaline and noradrenaline in a mixture depends on differences in the intensity of the fluorescence Cohen and Goldenberg (1957) have devised a method for determining both catecholamines with chromatographic separation — a method which has proved reliable in the hands of the present author

Determination of blood glucose concentration The method originally described by Huggett and Nixon (1957) was used in the modification described by Kramer Jacobsen (1960) and Hansen (1962) The analysis was carried out on whole blood after precipitation with perchloric acid

TABLE I Output of catecholamines from the left adrenal medulla in intact sheep after injection of insulin

Experiment	Time after inj of insulin	Blood glucose conc.	Adrenaline	Noradrenaline	$\frac{A}{A+NA}$
no	hours	mg	ng/kg min	ng/kg min	%
4	58	41	183	11.2	94.3
	1.25	28	127	17.1	88.2
	1.58	20	148	33.0	83.1
	2.14	27	145	19.6	87.9
	2.39	25	155	17.1	90.0
5	1.17	46	3[illegible].4	0.0	100
	1.48	37	43.1	0.0	100
	2.57	27	43.4	8.7	83.2
	3.30	26	32.8	5.8	85.0
6	1.22	23	14.1	22.9	38.1
	2.26	29	40.3	9.6	80.8
	2.53	34	50.9	13.2	78.2
[illegible]	2.10	31	92.0	2.7	97.1
	2.30	29	11	4.0	96.6
	2.55	33	112	5.8	95.3
24	2.00	2[illegible]	148	34.4	81.1
	2.25	41	144	66.0	68.6
25	1.28	20	122	17.4	87.5
	1.48	20	139	19.6	8[illegible].5
	2.05	25	162	18.5	89.8
2	1.20	35	10.4	0.0	100
	1.35	28	7.5	0.0	100
	1.40	27	9.4	0.0	100
29	1.00	24	15.9	5.[illegible]	[illegible]3.5
	1.05	27	18.3	5.1	[illegible]8.2
	1.25	2[illegible]	22.1	7.8	[illegible]4.0
30	40	28	110	23.8	82.1
	50	24	123	22.8	84.4
	1.07	19	104	15.4	87.4
32	57	6	30.[illegible]	1.9	94.3
	1.09	6	158	9.9	94.0
	1.20	6	25.3	2.0	92.[illegible]
		Average	83.2	14.2	86.0

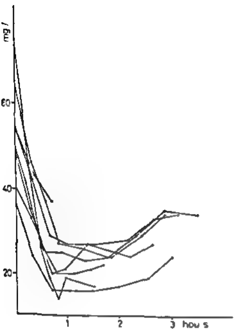

Fig 2a

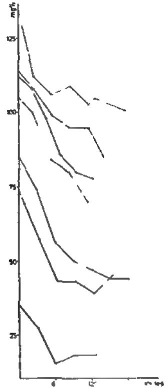

Fig 2b

Fig 2a Rate of fall of blood glucose concentration in the intact sheep after intravenous injection of insulin (2 IU/kg) at zero-time

Fig 2b Initial blood glucose concentration and rate of fall after intravenous injection of insulin in decerebrate sheep Decerebration was performed 1—2 hrs before insulin was given Zero-time Injection of insulin (5—10 IU/kg) Abscissae Time after injection of insulin Ordinates Blood glucose concentration (mg/100 ml)

Results

A *The response of the adrenal medulla to hypoglycemia in intact decerebrate and spinal sheep*

1 Animals with intact central nervous system The secretion of adrenal medullary hormones was followed in a series of experiments in which adrenal blood was collected before and after injection of insulin A rise in the output of adrenaline — compared with the output in the control periods — occurred in all experiments An increased output of noradrenaline was also observed in many instances but this effect was much smaller and less consistent

The output of catecholamines began to rise within 1/2—1 hours of the administration of insulin The onset was rather abrupt and it was easy to define when the adrenal medulla became activated Once activity had started it continued and the secretion

TABLE II Output of catecholamines from the left adrenal gland in decerebrate hypoglycemic sheep

Expt no	Time after decerebration (hours)	Blood glucose conc mg/100 ml	Adrenaline ng/kg/min	Noradrenaline ng/kg/min
8	2 30	15	19 2	6 9
	3 00	18	40 6	17 4
	3 30	18	66 1	22 4
27	23	27	24 4	19 0
	53	19	39 5	12 5
	1 18	19	30 9	23 4
28	28	38	164	30 8
	50	31	117	50 0
	1 14	28	169	21 2
30	41	24	124	67 5
		Average	86 9	31 6

TABLE III Output of catecholamines in decerebrate sheep before insulin was given

Expt. no	Time after decerebration (hours)	Adrenaline ng/kg/min	Noradrenaline ng/kg min
8	1 30	1 4	0 0
—	1 45	0 9	0 0
10	1 00	30 8	26 2
11	50	2 8	16 0
—	1 07	61 6	72 0
12	50	41 0	136 0
13	1 50	0 0	19 8
18	2 10	18 2	16 2
20	1 50	11 8	2 2
—	2 20	6 7	3 8
—	2 50	14 5	11 2
	Average	19 1	35 4

rate remained more or less constant during the following hours An example of this type of response is shown in Fig 1

Table I shows results from the individual experiments and it is seen that the amounts released varied considerably from experiment to experiment

The spontaneous secretion of catecholamines from the adrenal gland in anesthetized animals was determined before insulin was given. The average output of adrenaline

TABLE IV The output of catecholamines from the adrenal medulla in hypoglycemic spinal sheep

Expt. no	Blood glucose conc mg %	Adrenaline ng/kg/min	Noradrenaline ng/kg/min
22	24	0 3	0 0
	29	5 1	0 0
	30	9 2	0 0
26	[illegible]	10 1	3 4
	28	14 4	4.2
	34	24 3	14 5
	37	23 2	15 5
37	28	3 5	0 0
	21	3 1	0 0
	Average	8 7	3 1

was 5 0 ng/kg/min and of noradrenaline 2 7 ng/kg/min (6 expts) The secretion rate of noradrenaline is so small that it is at the lower margin of the sensitivity of the fluorimetric method and the average figure is therefore only an approximate indication of the secretion rate The spontaneous secretion rate was determined under circumstances similar to those existing during the hypoglycemic phase

b Decerebrate animals It soon became apparent that it was difficult to lower the blood glucose concentration in decerebrate sheep which showed a greater resistance to insulin than intact sheep While the average rate of fall of blood sugar in intact animals was [illegible] 68 mg % /min it was only 0.38 mg %/min in decerebrate animals Furthermore the initial level of glucose concentration was higher because the decerebration procedure in itself elicited a considerable increase in the concentration Yet another difficulty was encountered despite administration of large doses of insulin (10 I U / kg) *the blood glucose concentration tended — after an initial fall — to stabilize at a* level above that which was found in intact anaesthetized sheep Thus hypoglycemia in the ordinary sense of the word could scarcely be established These findings are shown graphically in Fig 2a and 2b

In order therefore to investigate whether a decerebrate sheep does respond to hypoglycemia the experimental procedure was changed The decerebration was carried out after the hypoglycemia was established In order to prevent the effects on blood sugar of adrenaline released from the gland during and immediately after the transection the adrenal effluent blood was collected for 5—6 min (during and after decerebration) The activity of the other gland was abolished by transection of the right splanchnic nerve These precautions made it possible to establish an experimental situation in which hypoglycemia existed in a decerebrate animal The results from experiments of this type are shown in Table II which shows that a decerebrate animal possesses the ability to activate its adrenal medulla

The figures in Table III show that decerebration in itself acts as a stimulus which initiates increased activity in the adrenal medulla but it seems permissible to conclude

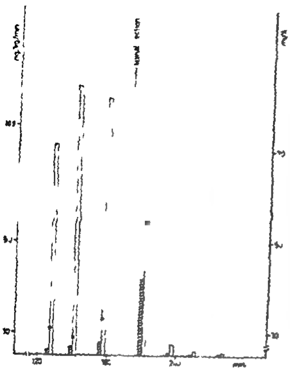

Fig 3 The output of catechol amines from the left adrenal medulla in a hypoglycemic sheep in which the spinal cord was transected during the hypoglycemic phase Insulin (7 1 IU/kg) was given intravenously at zero time The cord was transected 3 hrs and 15 min after the injection of insulin. After a short increase in the release of catecholamines following the transection the output falls to very low levels Abscissae Time after injection of insulin Ordinates Adrenaline ☐, noradrenaline ▣ blood glucose concentration ● — — — — ●

that when hypoglycemia supervenes a further increase in the secretion rate takes place. The average excretion of adrenaline in decerebrate animals with normal blood sugar was 19 1 ng/kg/min and in hypoglycemia 87 ng/kg/min. If the results are analyzed using the Wilcoxon ranking test for two samples (1945) the difference between the figures is found to be significant at the 5 % level if results from each animal are pooled If every figure in the tables is taken as a separate figure the difference is significant at the 1 % level The rate of secretion of noradrenaline does not change One parameter besides the glucose concentration of the blood is different in the control series and in the hypoglycemic decerebrate animals the time between decerebration and sampling is longer in the control series It is however unlikely that this difference can explain the difference in adrenaline secretion. In a long lasting control experiment on a decerebrate animal to which no insulin was given the secretion rate did not show any tendency to a definite increase or decrease within three hours

It was observed in several experiments that the output of catecholamines rose after injection of insulin despite the fact that the blood glucose concentration did not fall below "normal" levels The animals thus behaved as if they were functionally hypoglycemic. A low perfusion rate through the remaining part of the brain might contribute to this as the supply of glucose depends on the rate of blood flow as well as on the concentration of glucose in the blood

3 Spinal animals Attempts to investigate the adrenal medullary response to hypoglycemia in spinal animals met with some of the difficulties as described for the decerebrate animals The blood glucose concentration rose immediately after the interven

TABLE V Output of catecholamines from the adrenal medulla in hypoglycemic sheep immediately *following* decerebration (D) or transection of the spinal cord (S) The figures in brackets indicate the control levels (average output in three periods immediately before the transection)

Expt. no		Time after ablation min	Adrenal medullary output ng kg/min	
			Adrenaline	Noradrenaline
27	(D)	0–2	18.6 (9.1)	13.9 (0)
		3–6	73.5	20.0
		8–15	50.1	11.7
28	(D)	0–1	201 (18.8)	67.2 (6.9)
		2–3	1 050	367
		4–5	720	216
30	(D)	0–1	1,285 (11.9)	756 (20.7)
		2–3	1 000	323
		5–6	720	1 075
31	(S)	1–2	613 (10.7)	655 (7.9)
		2–3	147	373
		5–6	107	60
32	(S)	1–2	1 428 (71.3)	682 (4.6)
		2–3	314	262
		4–5	128	185

tion in the central nervous system, but the animals were not insulin resistant as the decerebrate animals were After insulin the blood sugar fell at almost the same rate as in intact animals (0 75 mg /min) The results in Table IV show the weak adrenal medullary response to hypoglycemia in these preparations The rate of secretion was so small that it was not possible to make any detailed analysis of the relative composition of the effluent.

Despite the occurrence of two strong stimuli each of which is well known to activate the sympathico-adrenal system (low blood pressure low blood sugar) only a negligeable output was demonstrated These preparations thus differed markedly from the decerebrate animals

Fig 3 shows as an example the reduction of the adrenal medullary output after the spinal section.

B *The immediate response of the adrenal medulla to decerebration or transection of the spinal cord.*
As described in the preceding section decerebration or transection of the spinal cord invariably resulted in an increased glucose concentration.

To see whether the transection itself elicits a vigorous output of catecholamines from the adrenal glands blood was collected from the adrenal vein in separate samples for

5—10 min after the intervention The results from these experiments are shown in Table V Regardless of whether the operation performed was decerebration or spinal section there was an immediate and very considerable rise in the output of catechol amines, followed by a tendency to a rather rapid fall

Discussion

The reported findings invite a discussion of the following questions

a) Which parts of the central nervous system can activate the adrenal medulla in hypoglycemia?

b) The relative composition of the adrenal effluent blood in hypoglycemia.

c) The insulin resistance of decerebrate animals

ad a) On the basis of the present observations there seems to be no doubt that regions of the central nervous system caudal to the hypothalamus respond to hypoglycemia and activate the adrenal medulla This need not be in contradiction to the generally held view that the adrenal medulla is activated from hypothalamic centres during hypoglycemia (Euler 1956) but the question must at any rate be raised as to the importance of these centres relative to other "sympathetic" centres in the nervous system The evidence pointing to the hypothalamus as the "trigger zone of adrenal medullary activity is, on the whole rather weak, based mainly on experiments with electrical stimulation through electrodes in the hypothalamus (Folkow and Euler 1954) and on Duner's experiments 1954) in which he showed a diminished resting' output of catecholamines when the level of the blood sugar was raised above normal (in cats) The conclusion that single nerve cells in localized regions of the hypothalamus have an activity which is correlated to variations in blood sugar concentration above the normal level is supported by Anand, Chinna and Singh (1962 Their arguments are however disputable as they find a common mode of reaction among all cells tested in such large regions as "centres Unpublished observations (Crone and Silver on the activity of single cells in the hypothalamus of the rabbit during hypoglycemia did not show the existence of well-defined centres of nerve cells with only one type of response to a given stimulus hypoglycemia

Whether the sympathetic cells in the isolated spinal cord can activate the adrenal medulla is a question which is not settled Tenney (1956 found that the response of the spinal cat to high concentrations of CO_2 was almost as great as that of the intact anesthetized animal and Comline and Silver 1961 found that foetuses of sheep with the spinal cord transected at C 1 did respond as effectively as intact foetuses to asphyxia. In the present series of experiments however the sympathetic centres in the spinal cord were found to respond very poorly if at all, to the stimulus of hypoglycemia. A reason for this finding may be that the perfusion of the spinal cord was inadequate because the blood pressure was low Voorhoeve 1960, thus found that in cats the small motor neurones stopped firing when the blood pressure fell below 50 mm Hg On the other hand it is well known, that all activity in the central nervous system does not stop at that level of blood pressure Barnes Joynt and Schottelius 1962 found that the membrane potentials of the big motor neurones in the spinal cord increased from 2 to 6 mV during spinal shock. This, they thought was due to removal of random synaptic bombardment. Until something is known about the detailed mechanism by which hypoglycemia activates sympathetic neurones it is however not profitable to speculate

about the apparent difference in the mode of reaction to hypoglycemia in different parts of the central nervous system. It must be mentioned, though, that Cantu *et al.* (1963) found in the dog that neither decerebration nor transection of the cervical spinal cord abolished the rise in adrenal medullary output in hypoglycemia, and it appears that these authors managed to keep the blood pressure above 80 mm Hg. Brooks (1931) came to conclusions similar to those in the present work. He showed that the reflex hyperglycemia (which is mediated via the adrenal medulla) persisted in thalamic and decerebrate cats, while it disappeared in spinal cats.

ad b) The present results support the statement that the adrenal medullary response in hypoglycemia is mainly an adrenaline response, but it should be emphasized that the response is not exclusively an adrenaline response. In almost all experiments a certain elevation of the secretion rate of noradrenaline was found. The adjustment of the composition of the medullary material depends obviously on a very coarse mechanism. One might expect a correlation between the degree of hypoglycemia and the intensity of the secretion of catecholamines, but it seems not to be so simple and it has not been possible, on the basis of the present experiments, to select any factor which determines the rate of secretion which, typically, is very variable from animal to animal.

ad c) The immediate explanation of the insulin resistance observed in decerebrate sheep would be that the secretion of adrenaline is of such a magnitude as to make mobilisation of glucose from the liver greater than the utilization and deposition of glucose in peripheral tissues. While this may be part of the explanation (the resting secretion of adrenaline in decerebrate animals is higher than in intact animals) it is certainly not the whole explanation, because there is no correlation between the ultimate level of blood glucose concentration and the rate of secretion of catecholamines. Donhoffer and MacLeod (1932) concluded from extensive investigations that increased glyconeogenesis took pace in decerebrate animals, a fact which may also explain the insulin resistance. Anderson, Rioch and Haymaker (1953) found that the ability to deposit glucose as glycogen after loading with glucose was diminished in chronic decerebrate animals. A mechanism which seems not to have been considered is the possibility that the decerebration *greatly reduces the circulation through the muscles* — an important region in the blood sugar lowering action of insulin.

I am greatly indebted to R. S. Comline, Ph. D., who introduced me to the physiology of the adrenal medulla and who generously helped me throughout my stay in Cambridge. Mrs. Marian Silver, Ph. D., gave me a very valuable introduction to fluorimetric technique for which I am most grateful. The work was supported by subsidies from the OECD Grants Committee and by the University of Copenhagen.

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Blood-Brain Barrier Alteration after Experimental Cold Injury of the Rabbit Brain, Indicated by Penicillin G in EEG and by Dye Tests

By

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Abstract

Flodmark, S. *Blood brain barrier alteration after experimental cold injury of the rabbit brain, indicated by penicillin G in EEG and by dye tests.* Acta physiol. scand. 1965. 63. 225—235. — Two previously known methods to indicate blood brain barrier alterations — intravital dye tests and EEG test after drug activation — were applied in parallel in 25 short- or long-term experiments on cold induced cerebral lesions in the rabbit. The method of cold application was a modification of that of Hass and Taylor, supplemented by a recording of the extradural temperature. A new type of permanent electrode for EEG recording is described. In 19 of 20 animals in which a structure-deranging lesion was induced, a concomitant blood brain barrier alteration could be demonstrated. In 11 of these 19 expts. a complete correlation of the two methods of barrier indication was obtained (8 animals inconsistent results). In 6 animals in which no blood brain barrier alteration was induced a concordance of negative results with the two methods was also seen. Three to four days was the maximum duration of blood brain barrier damage noted among the 13 animals in which the tendency to restitution of barrier function was studied. The results are in conformity with those of previous investigators and seem to justify the use of normally barred EEG activating drugs such as penicillin as detectors of blood brain barrier alteration. These drugs seem particularly suitable for iterative studies in living animals.

By means of a method for study of the interrelation between EEG and blood brain barrier phenomena (Flodmark and Steinwall 1963) the reversibility of some chemically induced blood brain barrier alteration as primarily shown by Broman and Olsson (1948) has recently been confirmed (Flodmark and Steinwall 1963 b). A similar tendency to restitution of a concomitant blood brain barrier alteration in physically induced structure-deranging cerebral lesions has been demonstrated in previous investigations by means of different acid dye indicators (Broman *et al.* 1949, Bakay *et al.* 1956, Klatzo *et al.* 1958, 1961, Åström *et al.* 1961) or by means of normally barred EEG activating drugs (Gonsette 1956).

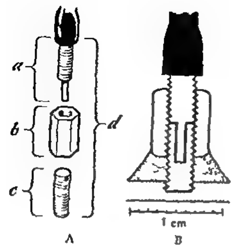

Fig 1 A The platinum plated metal electrode in detail
a the upper end of the metal screw with the connecting wire
b the plastic sleeve
c the lower end of the metal screw
B The electrode screwed into the bone and supported by plastic cement

In the present study on experimental cerebral lesion in the rabbit induced by local extracranial cooling these two different types of barrier function indicators were both applied to the same animals This series includes both short and long term experiments In the latter experiments any tendency toward restitution of barrier function was studied In both groups the functional state of the barrier was tested by means of the normally barred organic acid penicillin G as EEG activating drug and by means of acidic dyes (Congo red and/or trypan blue) enabling a postmortal visualization of a barrier damage existing intravitally at different stages of the cerebral lesion The principles of these barrier testing procedures have already been reported (Flodmark and Steinwall 1963 a and b)

Experimental procedures

In this series of experiments 27 young rabbits weighing 1 7– 2 7 kg were used As anesthesia during the intraosseous application of the electrodes and during the performance of the cerebral cold injury intravenous administration of pentobarbitone sodium (Nembutal Abbot) at a dosage of 0 03 g/kg was employed The duration of the anesthesia was approximately 2—4 hours

EEG-recording The electrodes consisted of platinum plated metal screws[1] see Fig 1 A and B The screws were applied intraosseously in bored holes with a depth of 0 5 mm without injury of the osseus lamina interna Further fixation and isolation was attained with plastic cement[2] applied around the holes filling the space between the bone and the plastic sleeve

A pair of electrodes was symmetrically placed over the hemispheres 5 mm anterior to the sutura coronaria i e just in front of the motor region Another pair was placed over the parieto occipital regions 12—15 mm behind the anterior ones The distance from the midline was about 5 mm A ground electrode was placed in the most frontal part of the skull Fig 2 shows a rabbit ready to be tested with the EEG machine Bipolar recording with an 8-channel transistorized Kaiser EEG machine was used During the EEG recording the rabbit was sitting or lying fairly comfortably in a basket which somewhat restricted the movements of the animal The basket was enclosed in a shielded box A control EEG in anesthesia was recorded before the induction of the cerebral cold lesion Within 30 min after the injury EEG recording was

[1] Planned and made in cooperation with the Dental Mechanical School Goteborg

[2] Clear Simplex Pentocryl (Autopolymerising Acryl Resin for Skull Closure) Dental Fillings Ltd London N 16

Fig 2 A Rabbit with 5 electrodes ready to be connected to the EEG machine for testing

reinstituted and continued up to about 4 hours In animals which were to be studied for several days EEG recordings in an unanesthetized state were made at regular intervals during the following days including usually both a resting record and a record during the penicillin injection (see typical experiments in the Results)

EEG classification The EEG in anesthetized or unanesthetized rabbits has been studied by several investigators see Longo (1962) Although principally symmetric slight asymmetries are frequently seen in both states The most common pattern when awake consists of regular 4—5 c/s waves but quite often dysrythmic patterns including 14—16 c/s episodes occur The main points vis à vis EEG in the present study were to estimate the degree of cold induced unilateral damage and its subsequent development and to determine the time after i v penicillin injection of any appearance of a significant number of spikes or sharp waves on the damaged side i.e to determine the focal spike time (FST)

The unilateral (left sided) changes induced by the cooling agent and observed in EEG were graded as I—III Grade I means an asymetric pattern consisting of somewhat lower amplitudes of the waves or the appearance of slow waves within 2—4 c/s frequency band slightly more often on the affected side Grade II means a pattern characterized by a preponderance of slow waves usually within 2—4 c/s frequency band on the affected side Grade III means a pattern where either the electrical activity is greatly depressed or a dominance of slow waves within 1—3 c/s frequency band is seen on the affected side

Method of production of cerebral lesion In this type of experiment it is desirable to avoid methods which include direct application of noxious agents on the exposed cerebral cortex since cortical exposure alone may induce uncontrollable impairment of blood brain barrier function (Samorajski and Moody 1957) Among the physical methods suitable for induction of neuronal damage and a concomitant alteration of blood brain barrier function cold application ad modum Hass and Taylor (1948) will satisfy this demand A simplification of their original technique was employed in the present study From a block of dry ice a core was made by means of a metal cylinder diameter 12 mm The cold injury was achieved by pressing the metal cylinder with the enclosed core of ice against the middle part of the os parietale on the left side the overlying muscles having been removed Pilot experiments showed that an application time of 1—2 min usually was necessary to induce a cerebral lesion which also included an alteration of blood brain barrier function as indicated by extravasation of trypan blue into the cerebral parenchyma The method however involves some possible sources of error the most important being the varying porosity of the ice unevenness of the contact surface between bone and ice and the variable thickness of the parietal bone in different animals The mode of procedure was therefore supplemented by means of simultaneous recording of the extradural temperature within the area in question The temperature was measured by means of an electrical thermometer[1] the applicator of which was inserted beneath the metal bone via a tangentially bored hole In this way a control of the cooling of the cerebral cortex within a local area was ascertained Preliminary experiments showed that lowering of the extradural temperature

[1] Electrical universal thermometer type TE3 with applicator type COR5 Elektrolaboratoriet Copenhagen (operating according to the thermocouple principle with an accuracy of 0 1 C within the range − 11 C — 46 C.

TABLE I Data concerning cerebral cold injury and the results of repeated tests indicating blood brain barrier state in 25 rabbits

Expt no	Cold injury: Applic time (sec)	Cold injury: Extradural temperature + C	Cold injury: sec	Macroscopic lesion	Within 1—2 hours: Dye test	Within 1—2 hours: EEG act test	1 day: Dye test	1 day: EEG act test	2 days: Dye test	2 days: EEG act test	3—4 days: Dye test	3—4 days: EEG act test	5—8 days: Dye test	5—8 days: EEG act test	Concordant results Dye EEG tests	Remarks
1	110	18—20	90	0	—	—									+	
2	120	22—24	50	0		—									—	
3	110	14 5—20	90	0	—	—									+	Very small Congo red spot right poster electr
4	120	14 5—17	110	0	—	—	—	—							+	
5	90	19—20	30	0	—	—	—								+	
6	60	13 5—19	45	1		—	—	—							+	
7	75	16—20	25	1											+	
8	100	19—21	35	3		+	+	+							+	
9	100	29—30	90	1	—	+	—	—							+	
10	100	18 5—20	70	1			—	—							+	
11	150	16—19	100	3	—		—	—							—	
12	100	27	40	3	—	+									—	
13	85	23—27	60	1	—	—									—	
14	90	13—15	45	2		+	—								—	
15	180	16—18 5	90	1	—	—							—	—	+	
16	65	14—15	45	1		—				+			—	—	+	
17	120	14—17 5	90	1	+	+		—				—	—		—	
18	60	17 5—21	30	(1)	—	—									—	Very slight trypan blue extravasation
19	90	technical troubles		3		—		—			+				—	Only small trypan blue spots
20	50	12—15	30	3		—									—	Hemorrhagic lesion Dye extravasation only centrally in lesion
21	110	19—20	90	(1)	—	—			—						—	Very slight Congo red extravasation
22	240	15 5—19	100	1	—	—	—	—							—	Very slight lesion microscopically
23	105	15—17	75	2		+		—			+	—			—	Very slight lesion microscopically
24	280	15—20	110	1	—	—					—	—			—	
25	120	technical troubles		?		+		—				+	—	—	—	

See next page

from about -36 C $\pm$ 1 down to $-18°$ C $\pm$ 3 for 30—90 sec. was usually sufficient to induce a focal alteration of neurons as well as damage to the blood-brain barrier function.

Testing of the state of blood-brain barrier functions During the run of the experiments the barrier function was tested by intravenous administration of two or three organic acids unable to pass from blood to brain under normal barrier conditions but can pass with any impairment of the blood brain barrier (Steinwall 1961) Among these acids crystallic penicillin was used to test the barrier function by means of EEG (Gonsette 1956 Flodmark and Steinwall 1963 a and b) One or both of the two acidic d es trypan blue and Congo red. were given in order to demonstrate the existance or absence of a barrier alteration at different stages of the cerebral lesion, as evident at inspection of the removed brain at the end of the experiment. Both of these dyes show a marked tissue affinity and poor diffusibility which make them well suited for staining purposes while the more easily diffus ble acid, penicillin, is more apt to bring about neuronal (EEG) influences after invasion through a defective barrier (Flodmark and Steinwall 1963 b The following agents and doses calculated per kg b w were used penicillin G 2—300 000 IE, Congo red 5—7 ml of a 1 solution and trypan blue 15—20 ml of a 1—2 solution. In principle the barrier testing procedures included 1 Congo red usually within 15 min. after induction of the cold injury (in 13 expts) 2 Penicillin G given the first time 30—60 min after the cold injury then at predetermined intervals during the following days (see Table I) 3 Trypan blue a few minutes before the termination of the experiments. Two typical experiments will be described in detail see Results

Histological examination In order to obtain a more precise understanding of the type, range and development of the cerebral damage the removed brains of 8 animals were examined histologically The brains were fixed in 10 neutral formol saline or in Carnoy s solution and embedded in paraffin. The following stains were used on paraffin sections 10 μ thick Luxol fast blue-cresyl violet according to Kluver Barrera and haematoxylin-eosin.

Results

The observations in relation to the experimental procedure are presented in the following descriptions of 2 expts

Typical experiment I (No 15 in Table I) *Blood brain barrier alteration manifest in the first days later restored* A nembutal anesthetized rabbit weighing 2 1 kg was used. By means of cold application the initial extradural temperature was lowered to $-$ 17.25 C $\pm$ 1 25 for 90 sec within an area of 12 mm in diameter frontoparietally on the left hemisphere The EEG record which before induction of cold injury showed a slight asymmetry Fig 3 A) was now changed to a distinct left-sided abnormality see Fig 3B (low amplitudes dominance of slow waves and absence of high frequencies on the left side) For postmortal indication of barrier damage in the early phase 12 ml of 1 % Congo red was administered i v 10 min after the cooling procedure. Somewhat more than one hour later 500 000 IE penicillin G was given i v in order to test the state of the barrier Two min later an epileptogenic activity appeared on the damaged left side see Fig 3C (recorded 15 min after penicillin injection) Three and a half hours later this pathological activity had vanished, see Fig 3D (the animal was out of narcosis Next day the

Acknowledgement is due to Dr Patrick Sourander Dept. of Pathology 1 the University of Goteborg for guidance in the histological evaluation of the lesions.

+ means appearance of spikes and or sharp waves in EEG from the damaged left hemisphere within 15 min after the penicillin injection except for expts no 12 and 25 in which a focal spike time (FST) of 3? and min respectively was seen.

— means absence of focal spikes and or sharp waves in connection with the penicillin injections.

TABLE 1 Data concerning cerebral cold injury and the results of repeated tests indicating blood brain barrier state in 25 rabbits

	Cold injury				Barrier tests and eating alteration											
					Within 1—2 hours		1 day		2 days		3—4 days		5—8 days			
Expt no	Applic time (sec)	Extradural temperature + C	sec	Macroscopic lesion	Dye test	*EEG act test	Dye test	EEG act test	Dye test	EEG act test	Dye test	EEG act test	Dye test	EEG act test	Concordant results Dye EEG tests	Remark
1	110	18—20	90	0	−	−									+	
2	120	22—24	50	0	−	−									−	
3	110	14 5—20	90	0	−	−									+	Very small Congo red spot right poster lectr
4	120	14 5—17	110	0	−	−	−	−							+	
5	90	19—20	30	0	−	−	−								+	
6	60	13 5—19	45	1		−	−	−							+	
7	75	16—20	25	1	−										+	
8	100	19—21	35	3		+	+	+							+	
9	100	29—30	90	1		+	−	−							+	
10	100	18 5—20	70	1			−	−								
11	150	16—19	100	3			−	−							[illegible]	
12	100	27	40	3	−	−										
13	85	23—27	60	1	−	−										
14	90	13—15	45	?		+	−									
15	180	16—18 5	90	1	−								−	−	+	
16	65	14—15	45	1		−				+			−	−	+	
17	120	14—17 5	90	1	+	+		−				−	−		+	
18	90	17 5—21	30	(1)	−	−									−	Very slight trypan blue extravasation
19	90	technical troubles		3		−		−			+				−	Only small trypan blue spots
20	50	12—15	30	3		−									−	Hemorrhagic lesion Dye extravasation only centrally in lesion
21	110	19—20	90	(1)	−	−			−						−	Very slight Congo red extravasation
22	240	15 5—19	100	1	−	−	−	−							−	Very slight lesion microscopically
23	105	15—17	75	2		+		−		−	+	−			−	
24	280	15—20	110	1	−	−					−	−			−	Very slight lesion microscopically
25	120	technical troubles		2		+		−				+	−	−	−	

See next p g

cerebral injury The resting EEG records in animals in which a positive penicillin effect was obtained could not be differentiated from those where no such positive penicillin effect was seen

Histological observations The eight histologically examined brains are representatives of the experiments where qualitative differences in barrier function or where inconsistent results were noted by means of EEG and dye tests

Group A No blood brain barrier alteration Histological findings Exp no 4 no signs of cerebral lesion

Group B A blood brain barrier alteration still existing at the end of the experiment Histological findings Expts 8 and 14 a sharply demarcated cortical necrosis with perivascular hemorrhages and *with migration of polymorphonuclear leucocytes from* the vessels

Group C Alteration of the blood brain barrier and restitution at the end of the experiment Histological findings Exp 15 a wedge shaped cortical necrosis invaded by microglia and adventitial histiocytes

Group D Experiments characterized by inconsistent results of barrier function tests Histological findings Expts 23 and 25 cortical necrosis invaded by scavenger cells *and further characterized by growth of new vessels from the meninges with proliferation* of capillary endothelial cells Expts 22 and 24 slight lesion with oedema and invasion of polymorphonuclear leukocytes Some proliferation of the endothelial cells of the vessels Tissue section of no 22 also showed ischemic changes of nerve cells in the second cortical layer

Discussion

The applied barrier indicators are subject to certain sources of error which may lead to misinterpretation of the results obtained Though suitable for purpose of staining trypan blue and Congo red or adequate for induction of neuronal effects penicillin these drugs fail as any blood borne substances will when circulatory stasis prevents their invasion into the damage parenchyma (See Broman 1949 Klatzo *et al* 1958) Such a mechanism probably acts in some experiments (no 19 and 20) in this series Furthermore the subsequent appearance of unilateral epileptic activity after intravenous injection of penicillin is perhaps not in itself conclusive for judging the status of blood brain barrier functions because several investigators have reported the appearance of epileptic activity after cold application to cerebral structures (Keith and Bickford 1954 *Morrell and Florenz 1957 Purpura et al 1958*) *On the other hand in the present* series such spontaneous epileptic activity in EEG was never observed in either the short term experiments or in animals examined repeatedly for a week Any unilateral epileptic activity noticed always occurred soon after the injection of the penicillin This close relation in time should indicate a causal relation The difference in spontaneous behaviour of cerebral cold lesion in the present series in comparison with those previously reported may be explained by the different techniques used for induction of the cold injury and possibly also by the different cortical regions exposed to cold

Another factor of importance as to the penicillin response in EEG is the number of reacting neurons within the barrier damaged area An ideal state for obtaining a maximal penicillin response in EEG seems to be a localized blood brain barrier damage without any simultaneous impairment of the nerve cells Under these circumstances the neurons are able to react optimally to the blood borne polar drugs Such a status

is probably more easily obtained when barrier damage is induced by means of certain chemical agents applied intravasally with special precautions (Flodmark and Steinwall 1962). In most other types of experimental cerebral lesions a combined alteration of the blood brain barrier and of nerve cells is present at least in some stages after induction of the lesion. Critical levels of the degree of the blood brain barrier alteration and of the number of vital nerve cells in the surrounding tissue may influence on the varying penicillin response obtained in different stages of one and the same lesion and in lesions of different degrees. Presumably these factors may contribute to the difficulties to interpret some of the results obtained.

The results of the present investigation show that a concomitant blood brain barrier alteration could be demonstrated by means of one or both of the applied barrier function tests in all but one of the animals (no 6) where a macroscopic cerebral lesion was achieved. This result is in conformity with those obtained by previous investigators (Broman *et al* 1949, Bakay *et al* 1956, Klatzo *et al* 1958, 1961, Åstrom *et al* 1961). A complete correlation of the dye tests and the penicillin test was obtained both in the 5 animals where no macroscopic lesion was present (no 1—5) and in 12 of the remaining 20 animals (no 6—17). The inconsistent results in 8 expts (no 18—25) require a few remarks. In 4 expts the EEG negativity after penicillin injection may be explained by the presence of a very limited damage as indicated by the small extent of the stained area (no 18 and 21) or by the results of the histological examination (no 22 and 24). In two experiments the explanation of the EEG negativity may be the hemorrhagic type of the lesion which admitted only a small spotty (no 19) or an isolated central (no 20) extravasation of dye into the necrotic lesion. In the remaining two experiments (no 23 and 25) no clear cut explanations of the inconsistent results can be suggested.

In the 13 animals of this series in which the duration of the blood brain barrier has been studied a similar tendency of reversion has been found as was noticed by the aforementioned investigators. The histological examination revealed proliferation of the endothelial cells of the vessel in 4 expts (no 15, 22, 24, 25) where restituted barrier function was observed according to the testing procedures used, while similar changes were absent in 2 expts (no 8 and 14) where the barrier was still defective. Since the capillary wall is a part of the structures which are responsible for the blood brain barrier phenomena, these proliferative changes of the vessels may be associated in some way with a restitution of barrier function. Whether this association is only a coincidence of time or if a more causal relation exists cannot be stated from the aforementioned few histologically examined experiments (*cf* Klatzo *et al* 1958).

The time of reversion as found by Broman *et al* (1949), Bakay *et al* (1956), Åstrom *et al* (1961) and the author ranges from 1—7 days, while Klatzo *et al* (1958, 1961) and Lee and Olszewski (1959) have reported experiments with defective barrier function persisting for 2—3 weeks, sometimes even 5 weeks. The varying duration of the blood brain barrier alteration, demonstrated by means of different indicator methods, apparently has many causes which however are of minor interest in this context. The main point is that acute structure-deranging cerebral lesions in animals include both an irreversible neuronal damage and an alteration of blood brain barrier function which however is reversible according to the aforementioned investigators. The present study supports this view and seems to justify the use of normally barred EEG activating drugs (as for instance penicillin) as detectors of blood brain barrier alteration. They are particularly suitable for iterative studies in living animals. The very potent effects on neuronal structures exerted by different polar drugs belonging to the same group of organic acids

as penicillin or to the group of organic cation compounds (Flodmark 1964) make these substances however less suitable for clinical studies of these problems An evaluation of a barbituric acid with low lipid solubility as barrier indicator in experimental and clinical work is in progress in this laboratory

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Acta physiol scand 1965 63 236—246

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Fusimotor Activity in a Flexor Muscle of the Decerebrate Cat

By

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Abstract

Jansen J K S and T Rudjord *Fusimotor activity in a flexor muscle of the decerebrate cat* Acta physiol scand 1965 *63* 236—246 — Fusimotor activity has been assessed from changes in the response of muscle spindle receptors There was very little background fusimotor activity to the anterior tibial muscle in the decerebrate cat To the ankle extensor muscles of the same preparations appreciable dynamic as well as static fusimotor effects were observed During stimulation of various spinal nerves fusimotor activation was more pronounced to ankle extensor than to the flexor muscle The flexor fusimotor effects could almost exclusively be ascribed to the static fusimotor system A release of dynamic fusimotor activity in the flexor muscle occurred after section of the spinal cord in the lower thoracic region The observations are discussed in terms of independent control of static and dynamic fusimotor systems

In the preceeding paper (Alnæs Jansen and Rudjord 1965) an attempt was made to analyse the fusimotor activity of the spinal cat in terms of activity in dynamic and static fusimotor systems For decerebrate cats it is known that there is a high level of background fusimotor activity in the extensor muscles (Hunt 1951 Granit and Kaada 1952 Kobayashi Oshima and Tasaki 1952 Eldred Granit and Merton 1953) This extensor fusimotor activity is evidently present in the dynamic as well as the static fusimotor system (Jansen and Matthews 1962 a) The object of the present study was to determine similarly the background fusimotor activity of a flexor muscle in the decerebrate cat and possible spinal reflex effects on this activity It will be shown that there is very little flexor fusimotor activity in this preparation and evidence of a descending tonic inhibition of the dynamic fusimotor system in flexor muscles will be presented

Methods

Eleven cats have been used Under initial ether anesthesia the anterior tibial muscle and lateral gastrocnemius or soleus were prepared for stretching Spindle receptor activity was recorded from thin dorsal root filaments All cats were decerebrated intercollicularly After determination

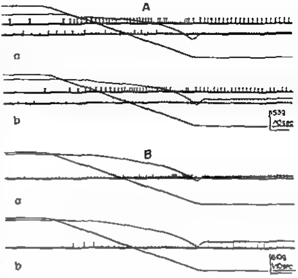

Fig. 1. Effect of de-efferentation on response of muscle spindle endings to standard stretch. A: Anterior tibial spindle receptors. Response of primary ending above, secondary ending below. a: Response with intact ventral root supply. b: Same endings after section of ipsilateral ventral roots L VI, L VII and S I. B: Response of primary ending in the lateral gastrocnemius. Same cat as A; a and b before and after section of ventral roots. Additional signals: a = muscle length (initially above) and muscle tension (initially below). Total extent of stretch 15 mm in all instances. The anterior tibial muscle was stretched to a length corresponding to full physiological extension. The lateral gastrocnemius was stretched to 4 mm less than full extension.

of the spindle response in the decerebrate state the spinal cord was cut at Th XII in eight of the cats. Details about the preparation, stretching of the muscles, recording procedures and nerve stimulations are given in the preceding paper (Alnæs *et al.* 1965).

Results

Background fusimotor activity. In the decerebrate cat there are usually only slight changes in the response of spindle receptors in the anterior tibial muscle to a standard stretch of the muscle after section of the ventral roots supplying the muscle. This is in striking contrast to observations made on the extensor muscle spindle receptors of soleus and of the lateral gastrocnemius. Examples from a typical experiment are shown in Fig. 1. The top records (Fig. 1 Aa) illustrate the response of an anterior tibial primary (above) and a secondary ending (below) recorded simultaneously during a linear extension of the muscle in a decerebrate cat. The response of the same two endings to a similar stretch after section of the ventral roots L VI, L VII and S I is seen in Fig. 1 Ab. There was no significant change in the behaviour of the endings. For comparison the response of a primary ending in the lateral gastrocnemius of the same cat is shown in Fig. 1 B. There was a marked decrease in the firing frequency of the receptor during the dynamic as well as the static phase of the extension after de-efferentation (Fig. 1 Bb) compared to that obtained while the ventral roots were still intact (Fig. 1 Ba). In the experiment from which the records of Fig. 1 were selected 15 spindle endings were studied before and after ventral root section. Five of these were primary and five were secondary endings of the anterior tibial muscle. The rest were primary endings of the lateral gastrocnemius. The anterior tibial endings all behaved like the pair illustrated in Fig. 1 A, exhibiting less than 10 impulses/sec change in dynamic index as well as static response. The response of all the 5 gastrocnemius endings was significantly changed after ventral root section. Their static response decreased by 20—40 imp/sec and their

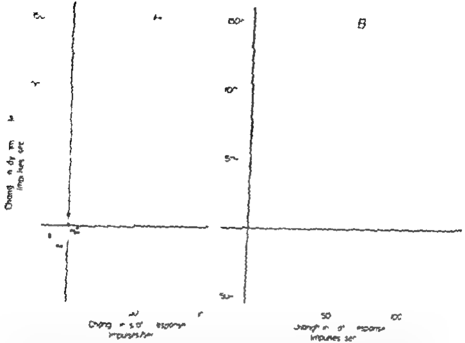

Fig. 2. Effect of de-efferentation on response of anterior tibial (A) and lateral gastrocnemius and soleus (B) primary endings to standard stretch. The changes in dynamic index are plotted along the ordinates and changes in static response along abscissa in both scatter diagrams

dynamic indices were decreased by 45—75 imp/sec for three endings and moderately increased for the last two endings (18 and 22 imp/sec).

The large changes in the responses of the gastrocnemius primary endings agree with the observations of Jansen and Matthews (1962 a) on soleus primary endings and are explained by the elimination of background fusimotor activity by the ventral root section. The lack of change in the response of the anterior tibial muscles suggests that there is much less background fusimotor activity to the flexor muscle spindles in the decerebrate cat. This is supported by the observations made in all 11 cats utilized in the present series of experiments. A summary of the observations on the effect of de-efferentation on all the primary endings studied is presented in the scatter diagrams of Fig. 2. The change in dynamic index is plotted along the ordinate and the change in static response along the abscissa. The observations on the 2? anterior tibial primary endings appear from Fig. 2 A and those of the gastrocnemius-soleus endings in B. It is seen that the response of the majority of the anterior tibial endings was not significantly affected by ventral root section.

Only two endings showed fairly large changes in response. It should be noted that these two endings did not behave regularly in this way. They both displayed signs of periodic changes in fusimotor activity. During the experiment, this could be heard in the loudspeaker as an apparently spontaneous increase in the discharge frequency of the ending. The two points plotted in Fig. 2 were obtained during such periods of increased fusimotor activity. These occurred at irregular intervals and lasted for about 1 min. Between such periods the response of the 2 endings was quite close to that obtained after ventral root sections. Such intermittent fusimotor activity was not observed for any of the other anterior tibial endings.

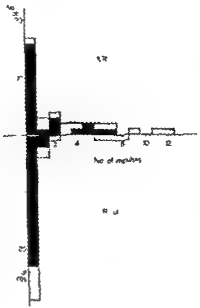

Fig. 3. Histogram of effect of ventral root section on the firing during release of stretch of anterior tibial primary (filled columns) and secondary endings (cross hatched columns). Number of units observed along ordinates. Number of impulses fired during release of standard stretch along abscissa. Observations with intact ventral roots are shown above the abscissa. Observations on the same units after de-efferentation are plotted below the abscissa. Total number of units [illegible] primary endings, 17 secondary endings.

Some anterior tibial endings displayed small changes in response after ventral root section which may be interpreted as a low level of background fusimotor activity. This did not manifest itself as an appreciable change in the mean frequency of firing during the stretch but as an irregularity of discharge and a tendency to discharge a few impulses during the release of the stretch. The observations of firing during release are illustrated in the histogram of Fig. 3. Each unit has been placed along the abscissa according to the number of impulses discharged during the release. The observations with intact fusimotor supply have been plotted above and those obtained after section of the ventral roots below the abscissa. Of the [illegible] primary endings 9 fired one or more impulses during release with fusimotor support. After ventral root section only two endings fired a single impulse. The observations for secondary endings are also illustrated in Fig. 3. As the primary endings they exhibited a reduced tendency to fire during the release of the stretch after ventral root section. No ending fired more impulses during release after de-efferentation.

In contrast to these weak fusimotor effects on flexor spindles the response of extensor spindle receptors was often dramatically changed by ventral root section (Fig. 2 B). There were large changes in the dynamic index as well as the static response of the majority of the endings and as pointed out previously (Jansen and Matthews 1962 a) there was no simple relationship between the changes in these two parameters exhibited by any one ending.

In conclusion it may be stated that in a series of decerebrate cats in which there was convincing evidence for dynamic as well as static fusimotor activity to the extensor muscles the slight effects on flexor spindle endings of interruption of fusimotor supply suggested only a low level background activity in flexor fusimotor neurones.

TABLE 1. Number of spindle receptors activated initially during stimulation of various spinal nerves. Numbers in brackets give the total number of endings studied for each nerve. X, contralateral; I, ipsilateral nerves

Nerve stimulated	Anterior tibial endings: Primaries	Anterior tibial endings: Secondaries	Gastrocnemius soleus: Primary endings
X Lat. popliteal	24 (35)	10 (14)	32 (34)
I n. saphenous	31 (35)	11 (14)	24 (34)
X median	27 (32)	12 (14)	19 (30)
I median	11 (20)	8 (11)	16 (22)
X sup. radial	7 (14)	2 (3)	[illegible] (11)
I sup. radial	[illegible] (27)	2 (4)	8 (11)

Effects of nerve stimulation. Fusimotor activity may be elicited in flexor as well as extensor muscle spindles by repetitive stimulation of various peripheral nerves in the decerebrate cat. In general, the effects on the anterior tibial endings were less powerful than those on the lateral gastrocnemius and soleus receptors. Classified as presence or absence of noticeable effect during stimulation of a particular nerve, the observations on all the endings studied are summarized in Table 1. Definite additional spikes in the discharge from a receptor at the initial length or an increase in the mean frequency of firing which could not be attributed to extrafusal relaxation were regarded as signs of fusimotor activation.

It appears from Table 1 that stimulation of a particular peripheral nerve could activate anterior tibial as well as gastrocnemius soleus endings. The threshold was usually the same for both muscles and was generally the same as that of ipsilateral flexor reflexes. Examples of the effects are shown in Fig. 4. It illustrates the response of two anterior tibial primary endings to stimulation of the contralateral lateral popliteal nerve (Fig. 4 A). Both endings started to discharge about 30 msec after the beginning of nerve stimulation. The records of Fig. 4 B are from the same experiment and show a comparable activation of 2 primary endings of the lateral gastrocnemius during stimulation of the same nerve at the same intensity. The intensity was above the threshold of the ipsilateral flexion reflex but below that of contralateral reflex effects. Similar observations have been made for the other nerves studied, and a particular responsive ending was usually activated from the contralateral lateral popliteal nerve as well as from the ipsilateral saphenous nerve. Thus, the present experiments with electrical stimulation of peripheral nerves do not indicate any obvious signs of a reciprocal organization of spinal fusimotor reflexes.

The lack of reciprocal fusimotor effects in the present investigation is to some extent different from the earlier observations of Hunt (1951) and Eldred and Hagbarth (1953) on spinal cats. That this should be due to a specific difference between the spinal and decerebrate preparations is considered unlikely in view of the similar absence of reciprocal fusimotor effects in the preceding study on spinal cats (Alnæs *et al.* 1965). However, Hunt (1951) and Eldred and Hagbarth (1954) employed physiological stimuli to elicit the reflex effects and this factor may explain the difference in results. Unfortunately, such stimuli were not suitable for the present experiments requiring stable and maintained fusimotor activity.

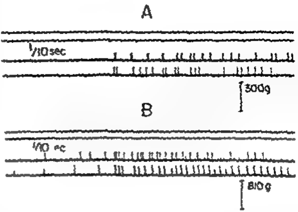

Fig. 3. Initial fusimotor activation during nerve stimulation. A. Response of two primary endings in the anterior tibial muscle to stimulation of the contralateral lateral popliteal nerve at 40 impulses/sec and an intensity twice the threshold of ipsilateral flexion reflexes. B. Response of two primary endings in the lateral gastrocnemius of the same cat to same frequency and intensity of stimulation of the contralateral lateral popliteal nerve. Nerve stimulation indicated by slight wobble on the top trace.

A reasonable number of anterior tibial secondary endings were studied during nerve stimulation. As a rule they were excited in the same way as the primary endings (Table I). This agrees with the observations on primary and secondary endings in the soleus of the decerebrate cat (Jansen and Matthews 1962 b) but is in contrast to the observations on spinal cats which on the whole are characterized by an absence of fusimotor effects on secondary endings (Alnæs *et al.* 1965).

All nerves stimulated in the present experiment could induce activity in the anterior tibial as well as the gastrocnemius soleus muscle spindles. Usually the anterior tibial spindles were excited equally by the contralateral lateral popliteal nerve and the ipsilateral saphenous nerve. The extensor spindles were on the whole more powerfully excited from the contralateral than from the ipsilateral hindlimb nerve but the difference was generally not marked. Regarding the forelimb nerves anterior tibial spindle activation was most frequently and powerfully evoked from the contralateral median nerve (Fig. 6). For the extensor spindles the difference between the various forelimb nerves was not that marked and the ipsilateral median nerve appeared to be more effective for extensor than for flexor spindle activation. At present however these differences between the various forelimb nerves do not lend themselves to an interpretation in terms of the possible functional significance of the effects.

As mentioned above the effects of nerve stimulation were as a rule more pronounced on the extensor than on the flexor spindles in the decerebrate cat. Another and more striking difference in the fusimotor activity of the two groups of spindles was revealed when the time course of the fusimotor reflex effects was considered. In the gastrocnemius soleus muscle spindles long lasting and apparently fairly stable fusimotor activation was achieved during continuous stimulation of the various peripheral nerves. In the anterior tibial spindles on the other hand the fusimotor activation usually declined quite rapidly and was only partly or not at all maintained during continuous nerve stimulation. This makes the interpretation of the fusimotor reflex effects on dynamic stretches more difficult. Quite often the fusimotor activation did not last long enough for the completion of the test extension. This is quite different from the situation in spinal cats in which longlasting and stable fusimotor activation was often observed in anterior tibial spindles (Alnæs *et al.* 1965).

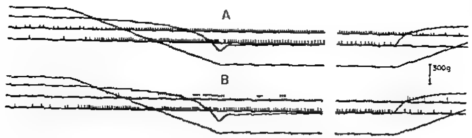

Fig 5 Effect of nerve stimulation on the response of anterior tibial spindle endings to standard stretch A Control response secondary ending above primary ending below B Response of same endings during repetitive stimulation of the contralateral lateral popliteal nerve at 40 impulses/sec Intensity 1 5 times threshold for ipsilateral flexion reflex Nerve stimulation initiated about 1 sec before beginning of record 0 7 sec of record cut out in the middle of extension Length and tension signals as in Fig 1 *Time marks 0 1 sec on tension trace*

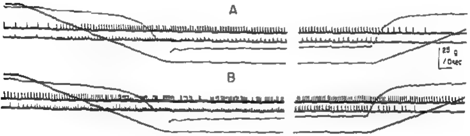

Fig 6 Marked effect of nerve stimulation on anterior tibial endings A Control response secondary ending above primary ending below B Response of same endings during stimulation of contralateral median nerve at 40 impulses/sec and intensity 1 5 times threshold for ipsilateral flexion reflex Stimulation initiated about 1 sec before beginning of record 0 5 sec of record cut out in the middle of static extension Length and tension signals 0 04 sec misaligned

However weak fusimotor effects on the dynamic behaviour of anterior tibial spindle endings were frequently observed and almost without exception the effects were quite characteristic An example of a typical finding is provided in Fig 5 The top records (*Fig 5 A*) show the discharge of a *secondary and primary ending of the anterior tibial* muscle during a control stretch in a decerebrate cat The modest effect on the response of the same 2 endings of stimulation of the contralateral lateral popliteal nerve soon afterwards appears from *Fig 5 B There* was a slight overall increase in the discharge frequency of the secondary ending *On the primary* ending the effect was too weak *to cause* a convincing increase in the mean frequency of firing but during stimulation the discharge of the receptor was definitely more irregular There was a small de rease in the dynamic index of the ending and a typical change in the res onse was the *firing of impulses* during the release

More obvious signs of fusimotor activatio ... ul ... ceptors are illustrated in Fig 6 The top records are ... e effect of stimulation of the contralateral median nerv ... *records* (Fig 6 B) There was a definite overall in ... he *secondary ending* The response of the primary

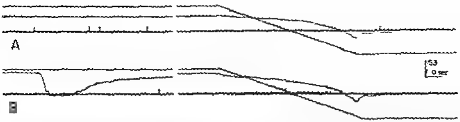

Fig. 7. Effect of spinalization on response of anterior tibial primary ending to stimulation of the ipsilateral saphenous nerve. A. Decerebrate cat. Beginning of nerve stimulation indicated by small wobble on length signal. B. Same ending after section of spinal cord at Th XII. Same intensity stimulation of ipsilateral saphenous nerve. Note release of ipsilateral flexion reflex in the anterior tibial muscle and more pronounced and maintained effects on spindle discharge. About 1 sec of record cut out in the middle between initial effect and test stretch in both A and B. Length and tension signals as in Fig. 1.

of the stretch. The discharge of this ending was now definitely irregular, there was a decrease in the dynamic index, and finally, the characteristic firing during release of the primary ending should be noticed. The records of Fig. 6 B is an example of one of the most powerful and sustained fusimotor effects found in the flexor spindles of the present experiments, and it should be pointed out that, compared to the effects on the extensor muscle spindles, they are not particularly impressive.

Thus, the effects of nerve stimulations on anterior tibial spindles in decerebrate cats can be summarized as follows: i The effects were weaker and less well sustained than those on the extensor spindles. ii There was a co-activation of primary and secondary endings. iii The discharge pattern of the receptors became less regular. iv An increased dynamic index of the primary ending was not observed. v Often this index was decreased, and finally, vi a discharge from the primary ending during release of extension was often induced.

Descending inhibition of flexor fusimotor activity. In the spinal cat, an increased response during the dynamic stretch and an increased dynamic index were the usual effects of nerve stimulation on the anterior tibial primary endings (Alnæs et al. 1964). Such dynamic fusimotor effects were not found in the flexor spindles of the decerebrate cat. This raises the question of a possible tonic inhibition of dynamic fusimotor activity in the decerebrate preparation. Therefore, an attempt was made to compare the behaviour of the same anterior tibial endings in the decerebrate and the spinal cat. An example of this is shown in Fig. 7. The top record (A) shows the response of an anterior tibial primary ending in a decerebrate preparation to stimulation of the ipsilateral saphenous nerve. There was a slight initial activation of the ending and no apparent flexor reflex in the anterior tibial muscle at the stimulus intensity employed. The initial signs of fusimotor activity vanished in less than 0.5 sec, and during the test stretch the response of the ending was indistinguishable from the earlier control stretch (not illustrated). The spinal cord was then cut at Th XII and approximately one hour later, the effect of saphenal stimulation at the same intensity was tested again. Now there was a release of an extrafusal flexor reflex in the muscle, corresponding to the release of flexor reflex activity in the spinal cat as described, for instance, by Job 1953 and Eccles and Lundberg (1959). At the same time, there was receptor discharge during the falling phase

of the *u* reflex and there was a definite increase in the discharge frequency of the receptor during the dynamic extension as well as an increase in the dynamic index. Thus there was a release of a dynamic fusimotor reflex in addition to the well known release of the *u* flexor reflex. Similar observations have been made in 4 of the 8 cats in which spinal section was attempted.

Spinal reflex inhibition of fusimotor activity. Reflex inhibition of fusimotor activity in decerebrate cats has been described in flexor as well as extensor muscle spindles (Hunt 1951, Voorhoeve and van Kanten 1962). The present experiments with the assessment of fusimotor activity from changes in the afferent discharge from receptors are not suitable for the study of fusimotor inhibitory effects because of the difficulty of excluding a small extrafusal contraction as the cause of a decreased spindle discharge. In addition the slight degree of background fusimotor activity in the flexor spindles of the decerebrate cat made this an unfavourable preparation for the observation of possible inhibitory spinal reflex effects. Even so, for a few anterior tibial receptors a slight reduction in discharge at the initial length was observed during stimulation of the contralateral lateral popliteal nerve. There were at the same time no detectable extrafusal contractions in the muscle and the effect was accompanied by a regularization of the discharge of the ending during stretch and a reduced firing during release. Although these effects were quite weak, it must be concluded, as appears entirely reasonable, that the electrical stimulation of peripheral nerve trunks as performed in the present study may have mixed excitatory and inhibitory effects on the flexor fusimotor neurons. In extensor spindles with their powerful background fusimotor activity definite signs of inhibition were observed during nerve stimulation. Thus in one decerebrate cat with a stretch reflex in the lateral gastrocnemius stimulation of the ipsilateral popliteal nerve resulted in the well known inhibition of the stretch reflex as well as a decreased discharge frequency of two primary endings. Such an effect must be interpreted as fusimotor inhibition. In addition suggestions of fusimotor inhibitory reflexes were seen occasionally as rebound activation of a spindle receptor at the end of nerve stimulation. No attempt was made to study these inhibitory effects in any detail.

Discussion

Certain features of the fusimotor effects on muscle spindle receptors appear to be particularly valuable for a qualitative distinction between activity in a dynamic and static fusimotor system. According to the work of Jansen and Matthews (1962 a, b) and Crowe and Matthews (1964 a, b) these are 1 The change in dynamic index 2 Co activation of primary and secondary endings 3 Presence or absence of discharge from primary endings during release of stretch and 4 degree of regularity of firing of a primary ending during fusimotor activation. At present these criteria are not rigidly quantifiable and as a rule any one of them would be insufficient in deciding whether a particular fusimotor effect is due mainly to one or the other of the two fusimotor systems. Taken together, however, such criteria should permit an identification of dynamic and static fusimotor effects. One problem to be expected is that fusimotor reflexes often affect both systems (Jansen and Matthews 1962 a) and so far it has not been definitely determined how activity in the 2 systems interact in their effect on the receptor properties (cf. Crowe and Matthews 1964 b).

With this background the present observations should now be considered. The low level of spontaneous fusimotor activity in the anterior tibial spindles of the decerebrate cat is well established (Fig 2). The slight effects of ventral root section were a reduced firing during release (Fig 3) and a regularization of the discharge. Both these effects suggest a small degree of static fusimotor activity in the flexor spindles of the decerebrate preparation. But this fusimotor activity was not sufficient to cause an appreciable increase in the mean frequency of firing of an ending during stretch. During

stimulation of various spinal nerves more convincing examples of selective static fusimotor activation were observed in flexor spindles of the decerebrate cat The relative lack of flexor fusimotor activity is probably an important factor for the lack of tonic stretch reflexes in the flexor muscles of the decerebrate preparation and the well known posture of this preparation is probably partly determined by the distribution of fusimotor activity in flexor and extensor muscles

The small degree of fusimotor activity in the anterior tibial spindles suggested that this activity might be depressed in the decerebrate cat This was supported by the finding of a release of dynamic fusimotor activation after spinal cord section Evidence of flexor fusimotor depression in decerebrate preparations have been presented also by Pascoe (1963) and Voorhoeve and van Kanten (1962) Such flexor fusimotor depression in decerebrates provides another example of the many well known linkages of α and γ motor effects and it raises the question of whether this fusimotor inhibition is controlled by the same bulbar structures as those responsible for the descending α flexor inhibition in the decerebrate cat (Holmquist and Lundberg 1961)

A reciprocal pattern of fusimotor activity appears to be present in the decerebrate cat with its high activity in extensor and low level of activity in flexor muscles Some examples of descending systems with reciprocal fusimotor effects have been reported (Shimazu Hongo and Kubota 1962) Considering the spinal reflex activation of the fusimotor systems surprisingly little evidence of reciprocal effects on flexor and extensor spindles was obtained in the present study The same applies to the preceeding investigation of fusimotor reflexes in the spinal cat (Alnæs Jansen and Rudjord 1965) This lack reciprocity in fusimotor effects suggests that the movements induced by spinal reflexes such as the present flexor and crossed extensor effects are not brought about as a follow up servo effect in the sense of Hammond Merton and Sutton (1956) That would in its simplest sense require fusimotor effects reciprocally organized like that of the α motoneurones In the study of the spinal fusimotor reflexes of ankle flexor and extensor spindles one is struck by the fact that these two groups of spindles are usually co-activated To introduce a teleological view one can suggest that the fusimotor activation is an anticipatory action to provide the appropriate dynamic and static sensitivity of both groups of receptors for the feedback required during the movements to be executed

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Acta physiol scand 1965 63 247—256

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Effect of Light and Moderate Exercise on Alveolar-Arterial O_2 Tension Difference in Man

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Abstract

HESSER C. M and G MATELL *Effect of light and moderate exercise on alveolar-arterial O_2 tension difference in man* Acta physiol scand 1965 *63* 247—256 — The effect of light and moderate exercise (400 675 and 700 kpm/min) on the alveolar-arterial O_2 tension difference (A—aD) in the sitting position was studied in healthy young adults by comparing the mean alveolar and arterial gas tensions determined over a 3 min rest period and over the ensuing 5th and 6th min of exercise Arterial gas tensions were obtained from continuous measurements of arterial pH oxygen saturation and temperature Alveolar O_2 tension was calculated by the alveolar gas equation substituting arterial for alveolar CO_2. At all three levels of exercise the arterial P_{O_2} increased significantly by about 5 mm Hg In the experiments at 675 kpm/min in which the respiratory exchange ratio (R) was also determined A—aD decreased from a mean value of 14 7 mm Hg at rest to 11 0 mm Hg during exercise In the experiments at 400 and 700 kpm/min where no ventilatory measurements were made in order to avoid interference with free respiration it was found that for the range of possible R values the A—aD remained unchanged or decreased during exercise The arterial O_2 deficit, i e the difference in O_2 content between pulmonary end-capillary blood and systemic arterial blood was calculated to decrease from a mean value of 0.27 vol % O_2 at rest to 0 18—0.22 vol % O_2 during exercise Likewise the calculated total venous admixture to the systemic blood flow decreased from a mean value of 4 7 per cent of cardiac output at rest to 1 7—1 8 per cent of cardiac output during exercise All the above changes are ascribed mainly to a reduction of the virtual shunt created by uneven distribution of alveolar ventilation to pulmonary capillary blood flow

Recent investigations have shown that the normal lung has such a high diffusing capacity that during air breathing at sea level no measurable oxygen gradient between alveolar air and end-capillary blood is created either at rest or during submaximal levels of exercise (TURINO *et al* 1963 STAUB 1963 Most of the alveolar arterial oxygen tension difference (A—aD) observed in these conditions can consequently be attributed to admixture of venous blood to the systemic circulation due to true veno-arterial shunt and uneven distribution of alveolar ventilation to pulmonary capillary blood flow

(virtual shunt) The magnitude of the A—aD and its components at rest and during exercise is of utmost importance for the evaluation of the effectiveness of the pulmonary gas exchange and has been studied extensively The results obtained so far have been contradictory both with respect to the magnitude of the difference at rest and its behaviour in response to exercise (for ref see Bartels *et al* 1955 Asmussen and Nielsen 1962) While most investigators have found the alveolar oxygen tension to increase slightly during exercise opinions have differed as to the direction and magnitude of the concomitant changes in arterial gas tensions However by using special recording techniques for the continuous measurements of arterial pH oxygen saturation and temperature it was shown in recent works from this laboratory (Matell 1963 Barr *et al* 1964) that in the sitting position there is a consistent increment of the arterial oxygen tension in the stable state of light and moderate exercise The present paper aims at a more detailed analysis of these observations with respect to factors involved in A—aD and pulmonary gas exchange during exercise A brief account for the changes occurring in A—aD has been given previously (Hesser and Matell 1963)

Methods

The basic data used for calculations of the alveolar and arterial gas tensions at rest and in the stable state (5th—6th min) of light and moderate exercise (400 625 and 700 kpm/min) were obtained from three series of experiments by means of continuous recording techniques (for technical details see Matell 1963 and Barr *et al* 1964) All measurements were made with the subjects in the sitting position After a Teflon catheter had been introduced into the right radial artery heparin (100—150 mg) was given i v and the subject was allowed to rest in the supine position for 30 min He then sat up on a bicycle ergometer and rested for another 10 min before exercising at a constant workload for 6 min The recordings were started at least 3 min before the work began Arterial pH (pH) and oxygen saturation (S_{O_2}) were continuously recorded by drawing blood via the Teflon catheter through a glass reference-electrode assembly and a cuvette oximeter at a constant rate of 8 ml/min by means of a roller pump The temperature of the blood passing the pH-oximeter assembly was measured by a thermistor unit in direct contact with the streaming blood There was no measurable drift of either the pH or the oximeter assembly during the experimental periods and the techniques used permitted the detection of changes in pH and S_{O_2} as small as 0 001 of a pH unit and 0 1 respectively CO_2 content O_2 content and O_2 capacity of arterial blood samples drawn anaerobically from the outlet of the pH-oximeter assembly were determined in duplicate by the manometric method of Van Slyke and Neill (1924)

In the first series of experiments (625 kpm/min) respiratory minute volume was recorded continuously by a rotary gas meter Expired air was collected over the same period as the blood samples and analyzed in duplicate for O_2 and CO_2 according to Scholander (1947) In the second and third series of experiments (400 and 700 kpm/min) the thoracic movements were recorded by means of a loosely attached pneumograph No other ventilatory measurements were made in these two series in order to avoid interference with free respiration All the continuously measured variables were inscribed simultaneously on photokymographic records A more complete description of the experimental procedure and the methods used has been given in previous papers (Matell 1963 Barr *et al* 1964)

For calculations of arterial gas tensions the individual pH and S_{O_2} records were first reduced to time averages over the last 3 min of rest prior to exercise and over the 5th—6th min of exercise The recorded pH values were corrected to the actual body temperature to blood tem

perature by using a correction facto of 0 0147 per degree C (Rosenthal 1948) For this p irpose the rectal temperature was followed continuously in the first series of experiments by means of a thermistor unit In the two other series the in vivo blood temperature was assumed to be 37 0 C at rest whereas the measured increment n blood temperature in the 5th and 6th min of exer cise averaged 0 3 C at both loads Arterial O tens on (P) was computed from the values for pH (corrected) S_{O_2} and blood (body) temperature using the line charts of Severinghaus (1958) *Arterial CO tension (P_{aCO}) was calculated from pH 37 and plasma CO content by* means of the Henderson Hasselbalch equation The serum pK 37 for ca bonic acid was obtained from the nomogram of Severinghaus Stupfel and Bradley (1956) and the solub lit coeff cient for CO in plasma at 37 C was taken to be 0.521 The plasma CO content was computed from CO_2 content hemoglob n concentration So and pH of whole blood by us ng the nomogram of Van Slyke and Sendroy (1928) The P_{aCO} values thus obta ned were transformed to *in vivo* bl od (body) temperature as described by Bradley Stupfel and Severinghaus (1956) The effect e alveolar O tension (P_{AO}) (Riley *et al* 1946) was calculated from the alveolar gas equation substituting arterial for alveol r P_{CO_2} and R_E for R_A In the experi ments at 400 and 700 kpm/min where no ventilatory measurements were made P_{AO} was computed for th ee assum ed values (0 8 0 9 and 1 0) of the respiratory exchange rat o (R) The d fference between P_{CO} and P_{ACO} due to venous admixture was computed to be less than 0 4 mm Hg i the present experiments

The experiments at 675 kpm/min were performed o 1 7 subjects those at 400 and 700 kpm/ min on 8 subjects All subjects were healthy male students well acquainted w th the experi mental cond t ons and rang ng in age from 20 to 26 years (mean 22 3) in he ght from 174 to 190 cm (mean 180 7) and in we ght from 61 to 89 kg (mean 68 3) The physical work ng capacity at pulse rate 170 was determ ned according to Sjostrand (1947) and Wahlund (1948) and averaged 1 085 kpm/min (range 800—1 300) Barometric pressure and room temperature a eraged 756 0 mm Hg and 24 4 C in the experim ents at 625 kpm/m n and 763 8 mm Hg and 23 4 C in the experiments at 400 and 700 kpm/m n

Results

Mean values for measured and derived data from the experiments at 625 kpm/min in which the pulmonary gas exchange was also determined are given in Table I 4 In Table I *B* and *C* the mean values for observed and calculated data from the experi ments with free respiration (400 and 700 kpm/min) are presented While there was no or only a slight increase of S_O in the stable state of exercise P_{aO} showed significant increments of about 5 mm Hg at all three levels of work The quantitative discrepancy between the S_O and P_O responses is readily explained by the facts that pH decreased and the *in vivo* blood temperature increased during the work periods both changes causing a shift to the right of the oxygen dissociation curve With free respiration the decrement in pH was greater at the heavier work (700 kpm/min) The fact that pH decreased still more at 675 kpm/min can probably be attributed to the somewhat higher breathing resistance resulting from the external breathing system used in these experiments Th s should induce a slight suppression of ventilation and a consequent rise in P_{aCO} and fall in pH Support for this view is given by the observation that the increment in calculated P_{CO} was somewhat greater at 675 kpm/min than in the two other series of experiments

Since in the experiments at 675 kpm m n P_{AO} rose somewhat less than P_O it follows that the A—aD decreased from a mean value of 14 7 mm Hg at rest to 11 0

TABLE I Measured and derived data from experiments at (A) 625 kpm/min (B) 400 kpm/min and (C) 700 kpm/min Air breathing sitting position In (B) and (C) the last six columns show the influence of different assumed R values on derived parameters (with most likely R values in bold face)

	pH_a	S_aO_2 %	P_aO_2 mm Hg	P_aCO_2 mm Hg	$\dot{V}O_2$ l/min STPD	$\dot{Q}$ l/min	R	Eff P_{AO_2} mm Hg	$(A-a)D_{O_2}$ mm Hg	C_c-C_a vol %	$\dot{Q}_s/\dot{Q}_t$ %	$\dot{Q}_s$ l/min	$\dot{Q}(C_c-C_a)$ ml O_2 per min
A Experiments at 625 kpm/min (7 subjects)													
Rest[1]	7 428	96 09	87 0	37 9	0 31	5 6	777	101 7	14 7	30	5 1	29	1
	024	20	4 7	2 8	03	–	015	3 1	7 3	16	–	–	–
Exer	7 385	96 14	92 1	41 0	1 39	13 6	892	103 1	11 0	22	1 8	25	30
cise[2]	028	60	6 1	2 4	12	–	060	4 3	5 9	13	–	–	–
B Experiments at 400 kpm/min free respiration (8 subjects)													
Rest[1]	7 407	96 50	93 0	37 1	0 30	5 5	8	105 6	12 6	25	4 4	24	14
	037	33	4 9	3 7	–	–	9	109 6	16 6	31	5 4	30	17
Exer	7 388	96 72	98 3	38 7	1 13	10 8	8	103 4	5 1	10	9	10	11
cise[2]	036	28	4 9	–	–	–	9	107 7	9 4	18	1 7	18	19
							1 0	111 1	12 8	24	1 2	24	26
C Experiments at 700 kpm/min free respiration (8 subjects)													
Rest[1]	7 414	96 51	92 7	36 2	0 30	5 5	8	106 6	13 9	27	4 7	6	15
	016	26	5 0	5 3	–	–	9	110 6	17 9	33	5 7	31	18
Exer	7 379	96 58	97 8	38 0	1 75	14 6	8	104 3	6 5	13	1 1	16	19
cise	042	33	5 2	–	–	–	9	108 5	10 7	21	1 7	25	31
							1 0	111 8	14 0	27	2 2	32	39

[1] Mean values and standard deviations
Rest data refer to time averages over last 3 min of rest preceding exercise
[2] Exercise data refer to time averages over the 5th and 6th min of exercise
[3] Assumed values (see text)

mm Hg during exercise) In the experiments at 400 and 700 kpm/min P_{AO_2} was calculated for three assumed values of R (Table I B and C) The influence of R on calculated P_{AO_2} and A—aD at rest and during exercise is illustrated in Fig 1 in which the relationships between R observed arterial and calculated alveolar gas tensions have been graphically depicted on O_2—CO_2 diagrams Inspection of Fig 1 shows that for all R values that may possibly exist in healthy young subjects at rest and in the stable state of light and moderate exercise, the increase in P_{AO_2} was less at both

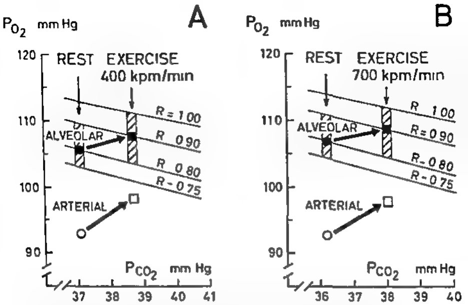

Fig 1 Alveolar and arterial gas tensions at rest and in the stable state of exercise (*A*) at 400 kpm/min and (*B*) at 700 kpm/min Air breathing sitting position The figure shows the influence of variation in R on the calculated alveolar P_O and hence on the A—aD Mean values of data from 8 subjects

levels of work than that observed in P_O Any increase in P_{AO_2} similar to or greater than that observed in P_{O_2} would require increments in the R values of 0 2 or more such great increments in R seem improbable at the work intensities studied in this investigation It may be concluded then that also in the experiments with free respiration the A—aD decreased during exercise If it is assumed that R increased by 0 1 i e showed similar increments as observed at 6?5 kpm/min the calculated decrease of A—aD amounts to 3—4 mm Hg at both work levels

Discussion

Factors influencing the determination of A—aD

Our findings that in the stable state of light and moderate exercise the mean values for A—aD were slightly reduced when compared to the mean values at rest are at variance with those of Lilienthal *et al* (1946) and Filley Gregoire and Wright (1954) who in moderately strenuous exercise found significant A—aD increases of 8—10 mm Hg and with results published by Bartels *et al* (1955) and by Asmussen and Nielsen (1960) who reported small and inconsistent increments in A—aD at similar levels of work The difference between these and our results can mainly be ascribed to different findings with respect to the magnitude and direction of the changes in arterial P_O occurring in response to exercise since in all these investigations the calculated effective alveolar P_O was found to increase While we observed consistent P_O increases of about 5 mm Hg both in light and moderate exercise the other investigators found no

change (Asmussen and Nielsen), a decrease (Lilienthal *et al.*, Filley *et al.*), or a slight but insignificant increase (Bartels *et al.*) in P_{aO_2} at similar work intensities.

As shown by Bartels *et al.* (1955) the variation of P_{AO_2}, P_{aO_2} and A—aD reported in the literature may in part be accounted for on the basis of the techniques used for measuring alveolar and arterial gas tensions. The above variation may, however, also be attributed to factors which only lately have been shown to have significant influence on actual or calculated gas levels. Such factors are the body position, the blood (body) temperature, and biological time variations in arterial gas tensions (cf. Barr *et al.* 1964). In a previous paper (Bjurstedt *et al.* 1962) it was shown that on shifting from the supine to the erect posture the A—aD increased significantly whereas P_{aCO_2} and P_{aO_2} decreased somewhat. These and other changes observed were ascribed to the influence of gravitational forces on the blood flow and its distribution in both the systemic and the pulmonary circulation. Hence, when studying the effect of exercise on P_{aO_2} and A—aD, rest and exercise data should be obtained with the subjects in the same posture. All the present data refer to the sitting position. If control data at rest are obtained in the supine position and exercise data in the upright position, the actual P_{aO_2} increase and the actual A—aD decrease evoked by exercise alone will presumably be underestimated.

The rising body temperature during exercise will introduce another error in the calculated A—aD if alveolar and arterial gas tensions are not determined at the actual body or blood temperature. The error introduced if neglecting the temperature effect on blood gas tensions can be estimated from the observations of Bradley, Stupfel and Severinghaus (1956) that a 1° C increment in the temperature of blood sealed in an anaerobic environment will raise P_{CO_2} by 4.4 per cent and P_{O_2} by 6 per cent in the physiologic ranges. Thus, if arterial blood samples are drawn anaerobically at 38° C from a subject breathing air at sea level and subsequently analyzed for P_{CO_2} and P_{O_2} at 37° C, P_{O_2} will be measured about 6 mm Hg too low and A—aD be calculated 8—9 mm Hg too high if due temperature corrections are not made. The estimated error in A—aD also includes the influence on calculated P_{AO_2} of a decreased inspired P_{O_2} at 38° C (about 0.5 mm Hg decrease) due to the higher intratracheal P_{H_2O} at this temperature. That P_{aO_2} increases during light and moderate exercise has recently been shown also by Holmgren and McIlroy (1964), who measured P_{O_2} of arterial blood samples at 37° C with an oxygen electrode (Severinghaus and Bradley 1958) and corrected the measured values to the arterial blood or esophageal temperature recorded at the time at which the blood samples were taken. In other reports dealing with changes in P_{aO_2} and A—aD during exercise no corrections for temperature changes seem to have been made.

The variation of P_{aO_2} and A—aD reported in the literature may in part also be ascribed to differences in blood sampling techniques. This matter was discussed in a previous paper (Barr *et al.* 1964). Using continuous recording techniques it was shown that in the sitting position spontaneous time variations occurred in arterial pH and S_{O_2} at half minute intervals or longer. These cyclic variations, which reflected corresponding cyclic changes in arterial P_{CO_2} and P_{O_2}, appeared in all 8 subjects studied and were quite marked in some individuals, especially in the resting condition. From the data presented the coefficient of variability in half minute P_{aCO_2} and P_{aO_2} values in the sitting position at rest can be calculated. In the single individual the coefficient of variability in P_{CO_2} averaged 2.2 per cent (range 0.8—3.2) and in P_{O_2} 3.3 per cent (range 1.3—7.2). Hence, if a blood sample is drawn over a rather short time, e.g. over a 30-sec period or less, its P_{CO_2} and P_{O_2} may not be representative for the actual

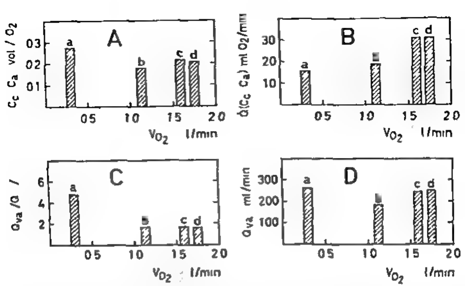

Fig. 2 Arterial oxygen deficit and total venous admixture in relation to oxygen uptake. Air breathing, sitting position. (*A*) arterial O_2 deficit in ml O_2 per 100 ml blood, (*B*) arterial O_2 deficit in ml O_2 per minute, (*C*) total venous admixture in per cent of cardiac output and (*D*) total venous admixture expressed as ml mixed venous blood per minute. In each graph *a* refers to resting conditions, *b* to exercise at 400 kpm/min, *c* to exercise at 675 kpm/min and *d* to exercise at 700 kpm/min.

in vivo mean values. To reduce the influence of time variations the arterial gas tensions in the experiments reported here were determined as time averages over a 3 min period at rest and over a 2 min period during exercise.

The calculated A—aD values at rest and during exercise in the experiments at 675 kpm/min, in which also ventilation measurements were made, were almost identical with those in the experiments with free respiration. It therefore appears that a slight increase in breathing resistance due to the use of an external breathing system has no appreciable effect on A—aD. To our knowledge the influence of increased external breathing resistance on A—aD has not been discussed or experimentally studied in earlier investigations.

Arterial oxygen deficit and total venous admixture

It is generally believed that the A—aD is composed of at least three components, due to (a) true veno-arterial shunts (anatomical shunt component), (b) uneven distribution of alveolar ventilation to pulmonary capillary flow (distribution component), and (c) diffusion limitations (diffusion component). Since the diffusion component is negligible in the normal subject breathing room air at sea level both at rest and during submaximal levels of exercise (Turino *et al.* 1963, Staub 1963), most of the decrease of A—aD observed in the steady state of light and moderate exercise must have been caused by changes in one or both of the two other components. While our data, being obtained from air breathing experiments only, do not permit separation of true venoarterial shunting from the unequal $\dot{V}_A/\dot{Q}$ effect (virtual shunt), the changes in total venous admixture ($\dot{Q}_{va}$) induced by exercise could be estimated.

Q is commonly expressed as a percentage of the cardiac output on the assumption that for a given experimental condition all fractions of the admixed venous blood have the same O_2 content Since this assumption is not correct and furthermore the a—$\bar{v}$ O_2 difference was not measured we have expressed the effect of venous admixture also as arterial oxygen deficit (Asmussen and Nielsen 1960) i e the difference between the O_2 content of pulmonary end-capillary blood (C) and that of systemic arterial blood (C) C_c was estimated as the O_2 content of blood being in complete equilibrium with P_{AO_2} and having the same pH O_2 capacity and temperature as observed in the systemic arterial blood The O_2 saturation of the end capillary blood (S_{O}) at prevailing conditions was determined from the line charts of Severinghaus (1958) and the oxygen in physical solution as 0 0031 P_{AO_2} where 0 0031 is the solubility coefficient for oxygen in whole blood expressed as vol / per mm P_O C was calculated from the observed values of S_{O_2} O_2 capacity and P_{O_2} In the experiments at 675 kpm/min the calculated arterial O_2 deficit decreased from a mean value of 0 30 vol / O at rest to 0 22 vol % O_2 during exercise (Table I 1) Similar decrements in arterial O_2 deficit were obtained in the experiments at 400 and 700 kpm/min (Fig 2 A) if it is assumed that R was increased by 0 1 during the work periods These results are at variance with those of Asmussen and Nielsen (1960) who found the arterial O_2 deficit to increase with increasing work load and O_2 uptake Likewise it can be shown that the data presented by Lilienthal *et al* (1946) Filley *et al* (1954) and Bartels *et al* (1955) would also yield greater values for the arterial O deficit during exercise than at rest

The arterial O_2 deficit was al o calculated in ml O per minute by multiplying the O deficit in vol % O_2 by the cardiac output (Q) expressed in deciliter per minute Values for Q at rest and during exercise (Table I) were derived from data on the relationship between Q and oxygen uptake (V_O) in the sitting position as given by Bevegård Holmgren and Jonsson (1960) This seems justifiable since their material was similar to our own with respect to age weight and physical working capacity In the experiments with free respiration (400 and 700 kpm/min) V_{O_2} was taken to be 0 3 l/min at rest whereas V_O during exercise was estimated by assuming a mechanical efficiency of 23 per cent and a caloric coefficient for oxygen of 4 As can be seen in Fig 2 B the calculated arterial O_2 deficit expressed in ml O per minute increased with the rate of O_2 uptake and hence with the work load The O deficits are rather small however and amount to about 5 per cent of V_O at rest and about 2 per cent of V_O during exercise Asmussen and Nielsen (1960) also found the O deficit in ml O_2 per minute to increase with V_O although the values from their air breathing experiments are about twice as large as the values presented here

The total venous admixture to the systemic arterial blood expressed as percentage of cardiac output was calculated from the standard shunt formula Q /Q = 100 (C −C)/(C −C$^-$) where C$^-$ is the O_2 content of mixed venous blood The a—$\bar{v}$ O_2 difference C —C$^-$ was obtained from V_{O_2} and Q according to the Fick principle Although the calculations of Q /Q were based on the fallacious assumption that the O_2 content was the same in all fractions of the admixed venous blood they should give a rough estimation of the magnitude of the changes occurring in the total shunt in response to exercise In the experiments at 625 kpm/min the calculated Q decreased from 5 1 per cent of Q at rest to 1 8 per cent of Q during exercise Similar decreases in calculated Q /Q were found in the experiments at 400 and 700 kpm/min (Fig 2 C) if assuming that also at these levels of exercise the R values were about 0 1 higher than

at rest These calculations thus suggest that light and moderate levels of exercise are accompanied by a marked reduction of that fraction of the total venous flow that reaches the systemic circulation via true and/or virtual shunts Bartels *et al* (1955) also found a reduced $\dot{Q}_{va}/\dot{Q}$ ratio during moderate exercise but the calculated reduction was rather small

The magnitude of the shunt flow (ml mixed venous blood per minute) equivalent to the total venous admixture was estimated through multiplication of $\dot{Q}_{va}/\dot{Q}$ by $\dot{Q}$ Although the $\dot{Q}_{va}/\dot{Q}$ ratio was markedly decreased at all three levels of exercise the shunt expressed as ml blood per minute was only slightly reduced at 400 kpm/min and remained essentially unchanged at the two heavier work loads (Fig 2 *D*) due to the increase in cardiac output

From the results of the above calculations it may be concluded that exercise of light and moderate intensity is accompanied by (1) a slight reduction of the arterial O_2 deficit expressed in vol % O_2 (2) a substantial rise of this deficit when expressed in ml O_2 per minute (3) a marked decrease of $\dot{Q}_{va}/\dot{Q}$ and (4) no appreciable change in the absolute size of the total shunt These changes as well as the decrease observed in A—aD must have been due mainly to changes in the true anatomical and/or virtual shunts As pointed out above our data do not permit separation of the anatomical shunt from the virtual shunt created by unequal $\dot{V}_A/\dot{Q}$ distribution Recent investigations (Lenfant 1963 1964) have shown however that at rest the real anatomical shunt is not as large as has been thought from measurements of the A—aD_{O_2} alone at high inspired F_O Thus in experiments with the subjects breathing 75 % O_2 at 2 6 atm Lenfant obtained evidence that in the sitting position the real anatomical shunt constitutes only about 25 % of the shunt estimated at sea level from the pure O_2 technique Since the contribution of the last mentioned shunt to the total A—aD is less than 50 during air breathing at rest (Ayres Criscitiello and Grabovsky 1964) it appears that under the same conditions only 10—15 % of the total A—aD and the total arterial O_2 deficit (vol % O_2) is due to real anatomical shunts Asmussen and Nielsen (1960) found on the other hand that both at rest and during exercise the shunt component estimated at high inspired P_O amounted to 20—25 of the total arterial O deficit at normal inspired P_{O_2} It can be concluded then that changes in A—aD and Q_{va}/Q occurring in response to light and moderate exercise are only to a minor part due to changes in the anatomical shunt component Since the diffusion component is negligible it follows that the above changes must be due mainly to alterations in the distribution component Most of the decrease in $\dot{Q}_{va}/\dot{Q}$ in the present work experiments can consequently be ascribed to an improved ventilation/perfusion distribution created by a reduction of the number of alveoli with low $\dot{V}_A/\dot{Q}$ ratios West and Dollery (1960) have demonstrated that in the erect position the distribution of $\dot{Q}$ improves during exercise It seems reasonable to assume that the distribution of ventilation is also improved during exercise because of larger tidal volumes with concomitant activation of previously nonventilated or underventilated alveoli

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Observations on 5-Hydroxytryptamine and Histamine Release from Rabbit Platelets[1]

By

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Abstract

Westerholm B *Observations on 5-hydroxytryptamine and histamine release from rabbit platelets* Acta physiol scand 1965 *63* 257—270 — Antigen glycogen n-decylamine and phospholipase A were shown to cause simultaneous release of 5-hydroxytryptamine (5-HT) and histamine from rabbit platelets *in vitro* There was no significant difference between the percentual release of 5-HT and histamine The release of 5-HT was studied in greater detail Release by antigen and glycogen both required the presence of plasma occurred at similar rates and showed distinct temperature optima around 37 C Heating the platelets to 47 C or plasma to 56 C produced an irreversible inhibition of such release Heparin, EDTA salicylaldoxime and the enzyme blocking agents ninhydrin (NH group inhibitor) and allicin (SH group inhibitor) inhibited release Reduced glutathione reversed the action of allicin High concentrations of sodium chloride blocked antigen induced release but did not significantly reduce release by glycogen. The results are considered to support the hypothesis that antigen and glycogen release 5-HT and histamine from rabbit platelets by the activation of similar release mechanisms and that enzymatic processes could be involved Release of 5-HT by n-decylamine and phospholipase A did not require the presence of plasma and could not be inhibited by treatments or agents found to inhibit or reduce release by antigen or glycogen The mechanism by which n decylamine and phospholipase A cause 5-HT and histamine release from rabbit platelets is considered to differ from that of antigen and glycogen

Antigen releases 5-hydroxytryptamine (5-HT) and histamine from rabbit platelets *in vitro* (Humphrey and Jaques 1953 1955) and *in vivo* (Waalkes *et al* 1957 Waalkes and Coburn 1959a 1959b 1960) That the two amines can also be released from these platelets by glycogen has been demonstrated by Waalkes and Coburn (1959b 1960) who suggested that release by antigen and glycogen involved the activation of similar release mechanisms This hypothesis is reinvestigated in the present paper The release of 5-HT in platelet rich plasma suspensions was studied under various experimental conditions using as active agents not only antigen and glycogen but also a surface

[1] A preliminary report on this study was presented at the Second International Pharmacological Meeting Prague August 20—23 1963 Biochem Pharmacol 1963 *12* Suppl. 211—212

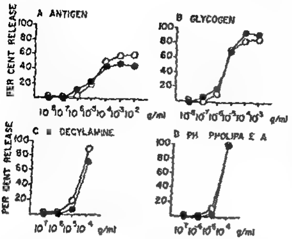

Fig 1 Concentration response curves of release of 5 HT and histamine in response to A antigen B glycogen C n-decylamine D phospholipase A Each point represents the mean percentual release of 5 HT O and histamine ● in duplicates In these experiments each sample contained 1 ml of platelet rich plasma 1 7 ml of buffered salt solution and 0 3 ml of buffered salt solution containing releaser

active agent n-decylamine and a lytic enzyme phospholipase A The latter has been shown to cause degranulation of rat mesentery mast cells by a mechanism characteristic of polymer-degranulating agents such as compound 48/80 and of antigen (Hogberg and Uvnas 1958 1960 Uvnas Diamant and Hogberg 1962, Uvnas and Antonsson 1963) The release of both 5 HT and histamine was determined in certain experiments to see whether release of the two amines occurred concomitantly in response to the releasing agents used

Methods

Rabbits (1 to 3 kg) of both sexes were used The effect of antigen (egg albumin) was studied on platelet concentrates obtained from rabbits previously sensitized to egg albumin The influence of glycogen n decylamine and phospholipase A was investigated on platelet suspensions from non sensitized animals

Sensitization procedure

Rabbits were injected intraperitoneally with 10 ml of a 10 per cent solution of crystallized egg albumin every day for 5 days and were used as indicated 2 to 4 weeks after the last injection

Preparation and incubation of blood platelets

Siliconized glassware was used when blood or platelets were handled in order to minimize spontaneous release of 5-HT and histamine

The animals were anaesthetized with pentobarbital Heparin 200 IE/kg was given intravenously as anticoagulant Blood was obtained by cutting the carotid artery and directing the blood flow into a centrifuge tube The blood was centrifuged at 700 g for 20 min at 0 5 C The supernatant plasma was carefully pipetted off Microscopic examination revealed that it contained a large amount of platelets and few or no leucocytes and erythrocytes Platelet rich plasma obtained in this way contained 4 7 ± 0 3 γ g/ml of 5 HT and 2 1 ± 0 7 γ g/ml of histamine as judged from determinations in 63 different experiments

One ml of platelet rich plasma suspension was added to conical centrifuge tubes each containing 0 8 ml of a solution of the following composition NaCl 1 54 × 10 M KCl 2 7 × 10 M CaCl 9 × 10 M and [illegible] per cent Sorensen phosphate buffer pH 7 2—7 4 (Na_2HPO_4 2 H_2O 6 7 × 10 M + KH_2PO_4 6 7 × 10 M) This solution is referred to below as the buffered salt solution The tubes were put into a water bath at 37 C Releasing agent dissolved in 0 2 ml of buffered salt solution was added to the diluted platelet rich plasma suspension The controls were mixed with an equal volume of buffered salt solution without releaser Unless otherwise stated incubations were run in duplicate

TABLE I Release of 5-HT and histamine from rabbit platelets induced by antigen glycogen n-decylamine and phospholipase A. Release values obtained from experiments in which both 5-HT and histamine were measured Values given as means and standard errors. In these experiments each sample contained 1 ml of platelet rich plasma 1 7 ml of buffered salt solution and 0 3 ml of buffered salt solution containing releaser

Releaser	Conc g/ml	Number of expts	Per cent release 5 HT	Histamine
Antigen	10	33	48 7± 4 4	42 0±3 8
	10 *	8	19 9± 8 1	19 9±5 1
Glycogen	10	4	78 3±15 5	85 1±7 4
	10 *	8	78 1± 5 9	68.2±7 0
	10	14	45 6± 7.5	29 7±5 2
n Decylamine	10	10	75 0± 8 6	60 1±9 0
	10	4	7 3± 4 3	? 3±1 4
Phosphol pase A	10	9	83 9± 6 7	81 4±7 3
	4×10	9	47 0± 9 6	3? 1±7 1
	10	4	9 1± 5 9	3 1±1 8

Incubation was continued for 15 min after which the tubes were cooled in an ice bath to stop further 5-HT and histamine release The tubes were centrifuged at 2 000 g for 10 min at 0 5 C. This treatment removed all platelets from the supernatant Since it was difficult to pour off the supernatant without stirring the platelets 1 ml of the platelet free fluid was carefully pipetted off and acidified, as was the residue in the tubes with 1 N HCl to obtain a final concentration of 0 1 N HCl The samples were kept at —17 C until 5-HT and histamine assay was performed

The experimental procedure was altered slightly when investigating the action of inhibitors The object was to allow the releaser to combine with plasma components before making contact with the inhibitor and the platelets Platelets were separated from the plasma by centrifugation at 500 g for 10 min at 0 5 C. Plasma was carefully poured off to minimize stirring of the platelets which were loosely packed at the bottom of the tube Platelets were washed twice by centrifugation with buffered salt solution and could when prepared in this manner be readily and uniformly suspended without any measurable leakage of 5-HT Platelet free plasma was obtained from blood of the same animal by centrifugation at 2 000 g for 10 min The platelets were resuspended in 1 ml of buffered salt solution containing inhibitor and preincubated for 15 min at 37 C before the addition of 1 ml platelet free plasma which had simultaneously been preincubated with releaser Incubation was then continued for another 15 min. The concentrations of inhibitors given in the text and figures refer to the final concentrations obtained after mixing the platelet suspension and plasma

Unless otherwise stated no spontaneous release of 5-HT or histamine could be demonstrated during preparation

The methods used for certain types of experiment are described in greater detail under Results

Assay of 5-HT and histamine

5-HT was determined spectrophotofluorimetrically by the method of Weissbach Waalkes and Udenfriend (1958)

Histamine was assayed on atropinized guinea pig ileum (atropine sulphate 1.5 × 10 M) according to Anrep Barsoum and Ibrahim (1947) The suspending medium Tyrode solution

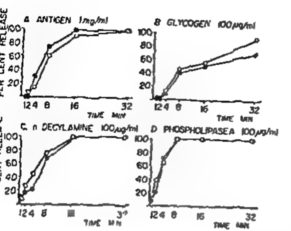

Fig 2 Time course of 5 HT and histamine release from rabbit platelets induced by A antigen 1 mg/ml B glycogen 100 µg/ml C n decylamine 100 µg/ml D phospholipase A 100 µg/ml Each point represents the mean percentual release of 5 HT ○ and histamine ● in duplicates In these experiments each sample contained 1 ml of platelet rich plasma 1 7 ml of buffered salt solution and 0 3 ml of buffered salt solution containing releaser Releaser was added at 37 C and samples were chilled rapidly after varying time intervals to 0 5 C

contained 1 µg/ml of 5 HT to abolish sensitivity of the ileum to 5 HT (Gaddum 1953) The specificity of the contractions was demonstrated by mepyramine block (mepyramine 5 × 10 M)

Releasers inhibitors and reactivators in the concentrations used did not influence the assay of 5 HT and histamine

All 5 HT and histamine values are given as the base

Error of method

The variation in the 5 HT release induced by 1 mg/ml of antigen in duplicates amounted to ± 5 0 per cent of the total 5 HT content as judged from duplicate tests of 33 different experiments The variation in the amount of histamine released in the same samples was ± 6 9 per cent of the total histamine content

Sensitivity of the test methods

The lowest concentrations of 5 HT and histamine which could be measured with the test methods used were 0 1 µg/ml and 0 03 –0 06 µg/ml respectively

Materials

Compound 48/80 prepared according to Baltzly *et al* (1949) and phospholipase A from bee venom were kindly supplied by Dr B Hogberg AB Leo Halsingborg Sweden

Allicin obtained from garlic by the method of Cavallito and Bailey (1944) was kindly prepared by Mrs I L Thon

The other substances used were obtained from standard commercial sources

Results

Release of 5 HT and histamine

Antigen glycogen n decylamine and phospholipase A were all found to induce 5 HT and histamine release from rabbit platelets The dose response relationships are shown in Fig 1 and Table I It can be seen that the percentual release of 5 HT is parallel to that of histamine In some experiments the release of histamine was less than that of 5 HT The difference was not however significant

Antigen 0 1—10 mg/ml produced no measurable release of 5 HT and histamine in diluted platelet rich plasma from non-sensitized rabbits (3 expts)

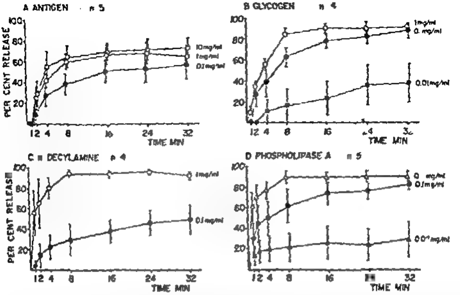

Fig 3 Time course of 5-HT release from rabbit platelets induced by A antigen 10 mg/ml □—□ 1 mg/ml ○—○ 0 1 mg/ml ●—● B glycogen 1 mg/ml ○—○ 0 1 mg/ml ●—● 0 01 mg/ml ■—■ C n-decylamine 1 mg/ml ○—○ 0 1 mg/ml ●—● D phospholipase A 0 2 mg/ml △—△ 0 1 mg/ml ●—● 0 01 mg/ml ▲—▲ Releaser was added at 37 C, and samples were chilled rapidly after varying time intervals to 0 5 C Each point represents the mean percentual release of 5-HT Vertical bars represent standard errors n = Number of experiments In the experiments only single tests of the release were performed

Time course of release

Fig 2 shows that 5-HT and histamine were released simultaneously from rabbit platelets in response to antigen glycogen n-decylamine and phospholipase A at 37 C With 5-HT the time course of release was also followed using different concentrations of the releasers (Fig 3)

Time curves for the release of 5-HT by antigen glycogen n-decylamine and phospholipase A were determined at various temperatures (Fig 4) A lowering of the incubation temperature delayed the appearance of measurable 5-HT release and resulted in a lower rate of release At 0 5 C none of the releasers produced any measurable 5-HT release after 64 min of incubation When the incubation temperature was raised to 47 C release by antigen was almost completely inhibited and that by glycogen significantly lowered (Fig 4A 4B) Release by n-decylamine and phospholipase A *on the other hand was faster at 47 C and was practically complete within 2 min* (Fig 4C, 4D)

To give a clearer picture of the temperature relationships between 5-HT release by antigen glycogen n-decylamine and phospholipase A the release values obtained with the four agents at different temperatures after incubation for 8 min are plotted in Fig 5 It can be seen that 5-HT release by antigen and glycogen was optimal around 37 C, while no optimum was obtained for n-decylamine and phospholipase A below 47 C

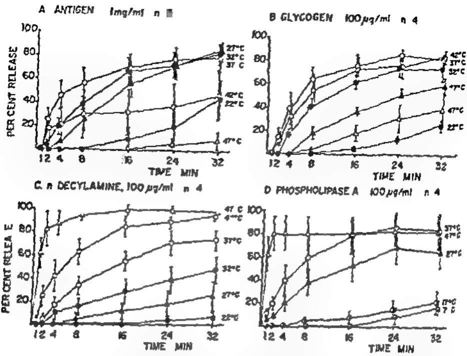

Fig 4 Influence of temperature on time course of 5 HT release from rabbit platelets induced by A, antigen 1 mg/ml B glycogen, 100 µg/ml C n-decylamine 100 µg/ml D phospholipase A, 100 µg/ml. Incubation temperature 7 C ◪—◪ 17 C ◐—◐ 22 C ■—■ 27 C, ▲—▲ 32 C, ●—● 37 C, ○—○ 42 C □—□ 47 C △—△ Releaser was added when temperature equilibration was achieved, and samples were chilled rapidly after varying time intervals to 0 5 C Each point represents the mean percentual release of 5 HT Vertical bars represent standard errors n = Number of experiments In the experiments only single tests of the release were performed

When the incubation temperature was 42 C or lower no spontaneous release of 5-HT could be demonstrated after incubation for 32 min At 47 C a measurable spontaneous release occurred in 3 expts out of 17 After incubation for 32 min it then ranged from 12 to 21 per cent of the total 5-HT in the platelet suspension In these experiments, the release values obtained with liberators were corrected for spontaneous release.

Effect of plasma on release

Humphrey and Jaques (1955) demonstrated that release of 5 HT and histamine from rabbit platelets by antigen and purified antibody occurred only in the presence of plasma. Later Barbaro (1961a, 1961b) showed that preformed antigen antibody complexes caused histamine release from rabbit platelets provided that plasma was present during incubation. The present experiments were designed to see whether release of 5 HT from rabbit platelets induced by glycogen n-decylamine and phospholipase A also required the presence of plasma. As a comparison, the 5-HT release induced by antigen was studied under the same conditions

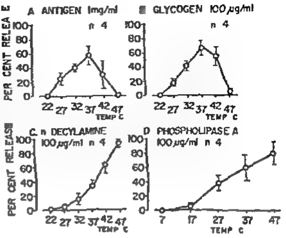

Fig 5 Influence of temperature on 5-HT release from rabbit platelets induced by A. antigen 1 mg/ml B glycogen, 100 µg/ml C. n-decylamine 100 µg/ml D phospholipase A 100 µg/ml. Time of incubation 8 min Each point represents the mean per centual release of 5-HT Vertical bars represent standard errors n = Number of experiments The values were obtained from the experiments shown in Fig 4

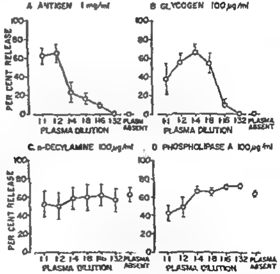

Fig 6 Influence of different plasma concentrations on 5-HT release from washed rabbit platelets induced by A antigen 1 mg/ml B glycogen 100 µg/ml C n decylamine 100 µg/ml D phospholipase A 100 µg/ml Each point represents the mean percentual release of 5-HT Vertical bars represent standard errors Number of experiments = 4

Platelets washed twice with buffered salt solution were resuspended in 2 ml of plasma that had been diluted to different degrees with the buffered salt solution Platelets and plasma were prepared from the same individual animal The releaser was added in a volume of 0 1 ml Fig 6 shows that release by antigen and glycogen required the presence of plasma while release by n-decylamine and phospholipase A did not. In some experiments high plasma concentrations seemed to exert an inhibitory action on release by antigen glycogen and phospholipase A although the finding was not statistically proved in the present series of experiments

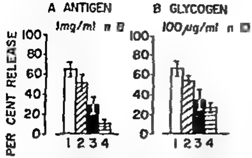

Fig 7 Release of 5-HT induced by A antigen 1 mg/ml B glycogen 100 µg/ml from rabbit platelets preincubated for 15 min at 37° C or 47° C The following platelet—plasma combinations were used
1 platelets preincubated at 37° C plasma preincubated at 37° C
2 platelets preincubated at 37° C plasma preincubated at 47° C
3 platelets preincubated at 47° C plasma preincubated at 37° C
4 platelets preincubated at 47° C plasma preincubated at 47° C

Each column represents the mean percentual release of 5 HT Vertical bars represent standard errors n = Number of experiments Significance of difference of means
A Antigen 1—2 not significant 1—3 $P < 0.01$ 1—4 $P < 0.001$
B Glycogen 1—2 not significant 1—3 $P < 0.02$ 1—4 $P < 0.001$

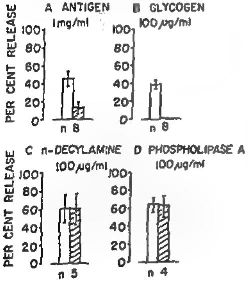

Fig 8 Release of 5-HT from washed rabbit platelets induced by A antigen 1 mg/ml B glycogen 100 µg/ml C n decylamine 100 µg/ml D phospholipase A 100 µg/ml in the presence of plasma preincubated for 30 min at 37° C ☐ or 56° C ▨ Each column represents the mean percentual release of 5 HT Vertical bars represent standard errors N = Number of experiments

Release after preheating of the platelets or plasma

Since the release of 5-HT from rabbit platelets by antigen and glycogen was inhibited at 47° C it was considered of interest to investigate whether the inhibition observed was due to a change in the platelets or in the plasma

In these experiments there were used platelets which had been separated from plasma by centrifugation at 500 *g* and resuspended in buffered salt solution The platelets were not washed since it was found that more than 50 per cent of the 5 HT present in platelets that had been washed twice with buffered salt solution was released spontaneously within 15 min at 47° C

The platelet suspension was preincubated for 15 min at 37° C or 47° C Platelet free plasma which had been obtained from blood of the same animal by centrifugation at 2 000 *g* was simultaneously preincubated for 15 min at 37° C or 47° C The platelet

TABLE II Inhib t on of 5-HT release from rabbit platelets induced by antigen and glycogen. n m = Release not measurable

Inhibitor		Antigen 1 mg/ml			Glycogen 100 μg/ml		
		Number of expts	Release in per cent of control w thout inhibitor		Number of expts	Release in per cent of control without inhibitor	
			Mean	Range		Mean	Range
Anhyd n	5 × 10 M	5	n m	—	4	n m	—
	10 M	4	10	n m —38	5	28	n. m —54
	5 10 M	4	42	n m —103	5	55	n m. —93
	10 M	4	97	95—100	5	99	90—112
Allicin	5 × 10 M	6	16	n m —59	5	24	3—46
	2 5 10 M	5	56	1—92	7	35	2—80
	5 × 10 M	4	102	97—115	3	100	97—108
EDTA	2 7 × 10 M	8	n m	—	8	n m	—
Salicylal	10 M	4	59	40—78	4	67	59—83
doxime	5 10 M	4	83	67—105	4	85	87—87
	10 M	4	100	93—111	4	95	88—100
Heparin	1 000 IE ml	6	[illegible]	n m —67	6	37	n m —64
	100 IE/ml	6	41	n m —87	6	94	69—119
	10 IE/ml	6	97	87—105	—	—	—

suspension and platelet free plasma were then kept at 37 C until temperature equilibration was achieved One ml of platelet suspension was then mixed with 1 ml of plasma as follows

1 platelet suspension preincubated at 37 C plasma preincubated at 37 C
2 platelet suspension preincubated at 37 C plasma preincubated at 47 C,
3 platelet suspension preincubated at 47 C plasma preincubated at 37 C
4 platelet suspension preincubated at 47 C plasma preincubated at 47 C

Antigen or glycogen was added and incubation continued for 15 min at 37 C Fig 7 shows that there was a significant reduction in 5-HT release when the platelets had been preincubated at 47 C The inhibition of release was still more pronounced when the plasma too had been preincubated at 47 C There was no significant reduction of the releas when platelets preincubated at 37 C were mixed with plasma preincubated at 47 C

From the same ind vidual animal unwashed platelets suspended in 2 ml of buffered salt solution and preincubated at 37 C or 47 C were incubated at 37 C with antigen or glycogen No measurable release of 5-HT occurred which proves that the amount of plasma remaining with unwashed platelets was insufficient to induce release

When platelets were preincubated at 47 C for 15 min a measurable spontaneous release of 5-HT was demonstrated in 9 expts out of 18 This ranged from 15 to 37 per cent Release values in these experiments were corrected for spontaneous release

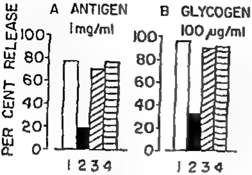

Fig 9 Influence of allicin and reduced glutathione on 5 HT release from rabbit platelets induced by A antigen 1 mg/ml B glycogen 100 μg/ml 1 Response to liberator alone 2 Response in the presence of allicin 2 5 × 10 M 3 Response in the presence of reduced glutathione 8 × 10 M 4 Response in the presence of reduced glutathione 8 × 10^{-4} M and allicin 2 5 × 10^{-4} M Each column represents the mean percentual release of 5 HT in duplicates

TABLE III Influence of different concentrations of sodium chloride on 5 HT release from rabbit platelets induced by antigen and glycogen Platelets were suspended in 1 ml of buffered salt solution containing different concentrations of sodium chloride whereafter 1 ml of platelet free plasma containing releaser was added Release value given as means Figures within brackets denote range n m Release not measurable

Releaser	Number of expts	Per cent 5 HT released Conc of NaCl in buffered salt solution added to rabbit platelets 0 08 M	0 15 M	0 23 M	0 31 M	0 46 M	0 62 M
Antigen 1 mg/ml	4	53 (32 100)	5) (38–100)	43 (19–100)	20 (n m –34)	5 (n m –19)	n m —
Glycogen 100μg/ml	5	44 (15–65)	68 (14–100)	69 (12–100)	78 (53–100)	66 (31–82)	47 (25–73)

In some experiments washed rabbit platelets were incubated with platelet free plasma which had been preheated for 30 min either at 37 C or 56 C At the latter temperature the complement required for immune hemolysis has been shown to lose its activity within a few minutes owing to destruction of C 1 and C 2 (Kabat and Mayer 1961 p 160) Fig 8 shows that 5 HT release by antigen was significantly reduced ($P < 0.01$) and that by glycogen completely inhibited in the presence of plasma preheated at 56 C Release by n decylamine and phospholipase A was unaffected

Inhibition of 5-HT release

Certain agents that are known to block different anaphylactic processes were investigated for inhibitory action on 5 HT release from rabbit platelets induced by antigen glycogen n decylamine and phospholipase A

Release induced by antigen and glycogen was inhibited in the presence of an amino group reagent ninhydrin and a sulfhydryl group inhibitor allicin (Table II) The inhibitory action of allicin was reversed by reduced glutathione (Fig 9) EDTA was

also found to block such release (Table II). A reduction in release was observed in the presence of salicylaldoxime and high concentrations of heparin (Table II). When the concentration of sodium chloride was increased in the buffered salt solution added to the platelets there was an inhibition of release by antigen but no significant reduction in release by glycogen (Table III).

Release of 5-HT from rabbit platelets induced by n-decylamine and phospholipase A was not inhibited in the presence of ninhydrin, 5×10^{-4} M, allicin 5×10 M, EDTA 2.7×10 M, salicylaldoxime 10 M and heparin 1 000 IE/ml (4 expts with each inhibitor). Nor was the release inhibited when the concentration of sodium chloride in the buffered salt solution was increased to 0.62 M (4 expts).

Discussion

The present observations that antigen and glycogen cause concomitant release of 5-HT and histamine from rabbit platelets confirm the work of others (for references see Introduction). It is evident that also n-decylamine and phospholipase A cause simultaneous release of 5 HT and histamine from rabbit platelets. Whether the two amines are released from the same or similar binding sites is not known. McLean, Nicholson and Hertler (1963), however, have suggested that 5-HT might be held in platelets in a different manner from histamine since reserpine, tyramine, chlorpromazine and imipramine were found to cause the release of a major portion of 5 HT from rabbit platelets without any marked decrease in the platelet histamine.

It is now well established that anaphylactic 5-HT release from rabbit platelets requires the presence of plasma. Not only antibody but also other plasma components seem to be involved in this release. Release of 5-HT and histamine by antigen and purified antibody or antigen antibody precipitates has thus been shown to occur only if plasma is present (Humphrey and Jaques 1955, Barbaro 1961a, 1961b).

According to Humphrey and Jaques (1955), Barbaro (1961b) and observations made in the present study, anaphylactic 5 HT and histamine release from rabbit platelets is dependent on the presence of a temperature sensitive plasma component, the activity of which is significantly reduced when plasma is preheated for 30 min at 56 °C. The heat-sensitive system in plasma does not seem to be identical with antibody, since heating of antiserum to 56 °C has been shown to have no great effect on the ability of subsequently formed antigen antibody precipitates to release histamine from rabbit platelets (Barbaro 1961a). Nor does it seem likely that the heat sensitive component of plasma is identical with the heat labile complement required in immune hemolysis since the heating of plasma to 56 °C only reduced its histamine releasing power but completely inhibited the complement activity (Barbaro 1961b).

When 1 mg/ml of antigen was added to a platelet rich plasma suspension, it was usually impossible to release all the 5-HT present in the platelets. No significant addition in the amount of 5-HT released could be obtained with an antigen dose as large as 10 mg/ml. This could be due to a certain relationship between antigen and antibody or other plasma components being required in the release.

Release of 5-HT and histamine from rabbit platelets in response to antigen does not seem to occur during the first minute(s) of incubation, although low amounts may have been liberated which could not be demonstrated with the test methods used. Observations by McIntire (1957) also indicate that there is no release during the first minute(s) of incubation. In his experiments, histamine release from rabbit blood in vitro did not

differ from the spontaneous release encountered in the controls McIntire also found that the preincubation of antigen with sensitized plasma for 3 up to 30 min before mixing them with normal blood did not shorten the lag period It therefore seems likely as he suggests that part of the lag represents the time required for proper orientation of the antigen antibody complex upon the platelet surface or for the initiation of some active process in the platelet

The curve relating antigen induced 5 HT release to temperature shows that there is a temperature optimum around 37 C At 47 C there occurs an irreversible inhibition of the release which according to the present results appears mainly to be due to a change in the platelets There is evidence however that the activity of plasma is also influenced

If the data presented in Fig 4 A are plotted as the velocity of antigen induced release against 1/T no straight line relationship is obtained This means that there is a change from one value of activation energy to another at different temperatures The findings indicate that the release involves different types of reactions which occur concomitantly or successively

The finding that antigen induced 5-HT release from rabbit platelets has a distinct temperature optimum suggests that enzymatic mechanism are involved The observations that ninhydrin and allicin inhibit the release are also compatible with such a suggestion since it is known that many enzymes depend on amino and sulfhydryl groups for their activity That allicin caused a specific block of the sulfhydryl groups is indicated by the fact that reduced glutathione restored the release of 5 HT

At present it seems unlikely that — as suggested by Humphrey and Jaques (1955) — a plasma protease is involved in the release since antigen induced 5-HT release from rabbit platelets is inhibited at 47 C while proteolytic mechanisms have been shown to be activated above 45 C (Ungar and Damgaard 1954) Nor does it seem likely that phospholipase A is involved in the release since its effects could not be inhibited at 47 C or by the agents found to block or reduce release by antigen

From the present findings it appears as if salicylaldoxime and high concentrations of sodium chloride — known to block the binding of C 3 in immune hemolysis (Mills and Levine 1958 Becker and Wirtz 1959) — inhibit antigen induced 5 HT release from rabbit platelets by a different mechanism Salicylaldoxime and high concentrations of sodium chloride were thus found to inhibit such release even though antigen and plasma components had been allowed to react with each other before the addition to washed platelets suspended in the buffered salt solution containing inhibitor

The inhibitory action of heparin on antigen induced 5 HT release from rabbit platelets as observed in the present study is in accordance with observations first made by Dragstedt Wells and Rocha e Silva (1942) that histamine release from rabbit blood elements into plasma is inhibited in the presence of heparin Johansson (1960) suggested that a possible role of heparin in anaphylaxis might be to prevent the onset of intravascular coagulation. However since the amounts of heparin required to cause a significant reduction of antigen induced 5-HT release from rabbit platelets by far exceeds the amounts necessary to prevent clotting it seems probable that heparin acts by means of another mechanism The inhibitory action of heparin might be due to its ability to form insoluble salt with protein (Jaques 1943) or to block the activity of certain enzymes (Horwitt 1940 Bergamini and Ferrari 1948)

There are certain similarities between antigen induced 5-HT release from rabbit platelets and anaphylactic histamine release from tissues of certain animals Antigen

induced histamine release from for instance rat and guinea pig tissues is thus inhibited in the presence of amino and sulfhydryl group blocking agents and EDTA and at temperatures above 45 C Furthermore antigen induced histamine release from guinea pig lung is inhibited in the presence of salicylaldoxime and high concentrations of sodium chloride (for references see Mongar and Schild 1962) It is conceivable that the mechanisms involved in antigen induced 5 HT release from rabbit platelets and in histamine release from certain tissues follow to some extent a similar pattern.

The findings in the present investigation give further support for the theory of Waalkes and Coburn (1959b 1960) that antigen and glycogen release 5 HT form rabbit platelets by the activation of similar release mechanisms Both processes thus require the presence of plasma take place at a similar rate depend on temperature in by and large the same way and can be inhibited by the same agents The only difference found so far between the two systems is in respect of their sensitivity to high concentrations of sodium chloride There is as yet no adequate explanation for this finding

Other polysaccharides have also been shown to release 5-HT or histamine from rabbit blood *e g* dextran and starch (Haining 1955 Waalkes and Coburn 1960) Amylopectin on the other hand has been found not to release 5-HT from rabbit platelets under the same experimental conditions as in the present investigation (Westerholm unpublished observations) Although there is yet no evidence in favour of such a suggestion the rabbit may have natural antibodies against certain polysaccharides Nelson and Lebrun (1956) have observed that sera from several species including rabbit contain antibody to starch

It is evident that release of 5-HT from rabbit platelets induced by n-decylamine and phospholipase A cannot be blocked by the agents or treatments found to inhibit release by antigen and glycogen It seems likely that n-decylamine and phospholipase A cause release of 5 HT and histamine from rabbit platelets by mechanisms different from those involved in release by antigen and glycogen

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From the Institute of Physiology, University of Lund, Sweden

Enzyme Activities in Various Organs of Hypophysectomized Rats and Rabbits

By

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Abstract

JACOBSOHN, D., S. LARSSON and A. NORGREN. *Enzyme activities in various organs of hypophysectomized rats and rabbits.* Acta physiol. scand. 1965. 63. 271—284. — The activities of nine different enzymes (glucose-6-phosphatase, combined activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, phosphohexose isomerase, aldolase, iso-citric dehydrogenase, lactic dehydrogenase, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase and glutamic acid dehydrogenase) were measured in liver, skeletal muscle, cerebral cortex, hypothalamus, adrenal cortex and thyroid gland of hypophysectomized rats and rabbits. The water and fat contents were determined in liver and muscle. DNA and protein were measured in all tissues. After hypophysectomy the activities of the Embden-Meyerhof enzymes were unaffected when the values were expressed per unit wet weight or per unit protein. When calculated on unit DNA the activities of almost all enzymes were in most tissues significantly decreased after hypophysectomy. This finding is due to the fact that except for cerebral cortex and hypothalamus in rabbits the DNA content of all tissues studied is significantly increased after hypophysectomy. The observed changes in enzyme activities and DNA content in the tissues are discussed in relation to hormonal and dietary influences on enzyme activities.

Ablation of the pituitary gland involves the removal of hormones regulating intermediary metabolic processes either directly or via endocrine target organs. Because of the absence of the hormonal actions the capability of the hypophysectomized individual to maintain homeostasis is impaired. Increased demands on the general metabolism such as necessary for growth of tissues cannot be met with. This situation has been clarified by manifold investigations of net effects of hormones on carbohydrate, protein and fat metabolism (*cf.* Aspinis 1962). *Little seems to be known about hormonal effects* on different steps in the intermediary metabolism. In most of the studies available so far, amino acids and enzymes were determined in selected tissues (*cf.* Knox, Auerbach and Lin 1956). Harper and Young (1959) found that glucose-6-phosphatase decreased in rat liver after hypophysectomy, and Snipes and Kostyo (196[illegible]) observed a depression

in the accumulation of utilizable amino acids in the diaphragm of hypophysectomized rats

The maintenance of homeostasis can hardly be achieved by adaptations of a single metabolic process and it seems desirable to clarify the conditions prevailing concomitantly in several different metabolic systems An attempt to approach the problem with this in mind was made by Fitch and Chaikoff (1962) The authors studied nearly all enzymatic activities involved in the metabolism of glucose in the liver of alloxan diabetic rats The results showed that the metabolic changes were not due to a single rate limiting enzyme

The considerations leading to the present investigation on hypophysectomized rats and rabbits were the same as those of Fitch and Chaikoff (1962) With regard to the metabolism of glucose and interactions with α amino acids the following enzymes were studied 1 Glucose 6-phosphatase (G 6 Pase) 2 Combined activities of glucose 6-phosphate and 6 phosphogluconate dehydrogenases (G 6 PDH) 3 Phosphohexose isomerase (PHI) 4 Aldolase (ALD) 5 Iso-citric dehydrogenase (ICDH) 6 Lactic dehydrogenase (LDH) 7 Glutamic oxalacetic transaminase (GOT) 8 Glutamic pyruvic transaminase (GPT) 9 Glutamic acid dehydrogenase (GlDH) The tissues studied were Liver skeletal muscle cerebral cortex hypothalamic tissue (rabbits only) adrenal cortex and thyroid gland (rabbits only)

Materials and methods

Experimental animals

Rats Fortynine males bred at this Institute were used A pelleted diet (FERROSAN Ltd Malmo) and tap water were freely available During about 3 weeks after demedullation of adrenals one percent saline was provided in addition to the tap water For the hypophysectomized rats the diet was supplemented with wheat white bread and fresh milk

At the age of about 4 weeks a group of 12 rats was unilaterally adrenalectomized the remaining adrenal gland being demedullated The reliability of the method was confirmed by microscopic examination of serial sections through demedullated adrenal glands of rats not presented here When the rats were 3 months old about half the number of rats of the groups with and without a demedullated adrenal gland were hypophysectomized by the parapharyngeal route (for control of completeness of hypophysectomy see Jacobsohn 1964) The rats were as far as possible randomly distributed within litters Tissues were obtained after decapitation of the rats a fortnight after hypophysectomy that is at the rats age of 3 1/2 month

Rabbits Twelve adult white males were used All rabbits were purchased from the same breeder and fed *ad libitum* with pellets (Forss Ltd Stockholm) mixed grain beetfor and tap water Occasionally carrots were given in addition To avoid the possibility of coccidiosis a sulfonamide derivative (Rigesol FERROSAN Ltd) was added to the drinking water during the first 3—5 days after the arrival of the rabbits at the institute

After a period of at least a fortnight at the institute 6 rabbits were hypophysectomized by the parapharyngeal route (*cf* Jacobsohn 1964) About 3 weeks after hypophysectomy with one exception at 15 days the rabbits were decapitated and tissues removed for study For the control of the completeness of the hypophysectomy the soft tissues around the base of the skull were removed as far as possible The sella turcica with adjacent tissues were dissected free fixed and decalcified during several weeks in Bouin s solution A block of tissue including the sella turcica and the median eminence of the tuber cinereum of the brain was then embedded in paraffin wax and cut into serial sections of 10 μ thickness After staining with haematoxylin eosin the sections were examined for residual hypophysial tissues (*cf* Jacobsohn 1964) The hypophysectomy was regarded as complete when the microscopic examination of the serial sections failed to reveal any anterior pituitary gland cells Rabbits which at the final control showed to have been incompletely hypophysectomized were excluded from the present material The body weight of the hypophysectomized rabbits remained fairly constant

Treatment of the tissues

Post mortem the tissue samples to be stu lied were removed as qu ckly as possible The samples ere then with the exceptions given below homogenized in ice-cold isotonic sucrose in an all glass homogenator Liver samples were taken from the centre of the major lobe in both species I the rabb t particular attention was pa d to avoid a rupture of the gallbladder or of any of the larger bile ducts. The muscle samples were taken from the caudal parts of the *longissimus d si* in the rabbit and from the gluteus region in the rat. The adrenal cortex was carefully dissected free from medullary tissues Demedullated adrenals were cleaned from surrounding tissues The thyro d glands of the rabb ts were dissected free from fat and connective tissue In both species the samples of the cerebral cortex were taken from the area just caudal to the s gmoid gyrus In the rabbit samples from the hypothalamus re its medial parts were also taken

Biochemical determinations

The *tissue p o in* content was determined accord ng to the method described by Ellman (19 2) based upon a modification of the Biuret react on ut lising changes in the ultraviolet spectrum accompanying the eaction The method presented d fficulties in determinat ons of protein in the adrenal cortex because of the occurrence of a pronounced opacity The method of Lowry *et l* (1951) was preferred in this tissue

Des xyribonucle ac d (DNA) was extracted from the tissue in most cases acco ding to the method described by Schneider (1945) or by Schmidt and Thannhauser (1945) The DNA content was determined either by the method described by Ceriotti (1952) or by Dische (1930) as mod fied by Racker (1957) At an early stage of the invest gat on extra tion and determina t on of DNA was made according to Scott Fraccastoro and Taft (1956)

Gluc se-6-phosphata e (G-6-Pase) act vity was determined as described by Schwartz and Bodansky (1961) a method based on the colorimetric neasurement of the u organic phosphorus liberated in 30 min from glucose-6-phosphate at pH 6 5 The *tissue phosphorus* was determined in the same way as the en yme but with the omiss on of glucose-6-phosphate Phosphorus was determined by the method of Fisk and SubbaRow (1925)

Comb ned activities of *glucose-6-pho phate* and *6-pho ph gluconate d hyd genase* (G-6-PDH) were determined according to the method by Glock and McLean (1953) essentially follow ng the modifications used by F tch Hill and Chaikoff (1959) As whole homogenates were used n the present study it was found necessary to prec pitate the proteins with ethanol and sodium sulphate to avo d opacity The comb ned activity of the d hydrogenases was also determined accord ng to Schmidt Schmidt and Wildhirt (1958)

Phosphohex se isomerase (PHI) was determined acco ding to the colorimetric procedure de scribed by Schwartz and Bodansky (1961)

Aldolas (ALD) was measured as described by Sibley and Lehninger (1949 based on a colorimetric measurement of the hydrazones formed by th triose phosphates by interaction with 2 4-d nitrophenyl hydrazine

TPN pecific iso-citric dehyd genase (ICDH) was determined ccord ng to the m thod described orig nally for serum by Ba on and Bell (1959 The tissue oncentration used here was 1 500

Lactic dehydrogenase (LDH) was d termined a cording to Cabaud and Wroblevsk (1958 based on the interaction of ketogroups w th 2 4-din trophen l hydrazin to form hydrazones The tissue homogenate was diluted to a f nal con entrat on of 1 2 000

Gl tamic-ox lac tic (GOT) and *glutam-c-pyruvic* (GPT *transaminases* wer determ ned olori metrically a cording to Re t nan and Frank l (1957 In these ases th tissue samples were homogenized in 0 1 M phosphat buffer at pH 7 Comp rison with samples homogen zed in isoton c sucrose sal n or water ga dentical results. The final d lutions of the h mogenates ere in all cases 1 500

Gl tamic acid dehyd genase GlDH ct ty was d termined ccord ng to Copenhave McShan and Meyer (1950) us ng sem carbaz d to obta n tru deh drogenase values When sem car ba de was omitted in reased Q_{O_2} al es were found probably due to ox dation of α keto-glutarate

W ter nd f t content was determined us ng pet oleum-ether to extract the fat in a Soxleth appa atus.

TABLE I. *Mean ± standard error of mean (number of observations) of activities of the enzymes indicated* The values are calculated per g wet weight. The data are expressed as follows gland determined according to Glock and McLean 1953) *PHI* mg fructose formed min, *LDH* μM pyruvate converted per min *GOT* and *GPT* μM glutamate formed The differences between intact and hypophysectomized rats have been calculated

	DNA	G-6-Pase	G-6-PDH	PHI
	Liver			
Intact	4.2±0.12 (24)	11.3±0.55 (15)	82.3±8.80 (17)	48.4±2.24 (14)
Hypophysect.	7.3±0.29 (24)	7.1±0.44 (11)	72.9±3.21 (18)	53.1±2.61 (12)[b]
	Muscle			
Intact	1.0±0.05 (25)	1.3±0.09 (11)	2.5±0.04 (10)	78.5±7.00 (11)
Hypophysect.	1.4±0.09 (24)	0.9±0.06 (10)[b]	3.0±0.05 (10)	78.5±6.21 (11)
	Cerebral cortex			
Intact	2.4±0.14 (11)	0.5±0.08 (10)	68±1.51 (8)	29.2±3.40 (8)
Hypophysect.	2.9±0.07 (13)[b]	0.5±0.04 (10)	40.6±2.47 (8)[b]	25.2±3.71 (8)
	Adrenal cortex			
Intact	7.3±0.89 (11	1.5±0.12 (10)	447.9±23.8 (10)	4.2±0.67 (8)
Hypophysect.	16.9±2.43 (10[b]	3.0±0.44 (10[b]	196.5±22.2 (10)	2.8±0.56 (8)

Results

The protein content of liver and muscle seemed to have a tendency to fall after hypophysectomy Significant changes were not found however *Desoxyribonucleic acid* (DNA) The amount of DNA in liver was determined with different methods of extraction, and with different colour reactions A difference between the extraction methods of Schneider (1945) and Schmidt and Thannhauser (1945 was not found The results obtained with the method of Ceriotti (1952) using indole and that of Dische (1930) modified by Racker (1952 using diphenylamine to react with desoxyribose did not differ However the method by Scott *et al* (1956 involving a great number of steps gave lower values. In addition it did not seem to be sufficiently specific for DNA

As may be seen from Table I the DNA content of the rat liver increased after hypophysectomy Table I also includes the values obtained from other tissues of normal and hypophysectomized rats. As for the liver the DNA content of these tissues was significantly higher in the hypophysectomized rats This was true irrespective of whether th

in Fig 1 — Rats
DNA mg G-6-Pase mg P released in 30 min G-6-PDH ΔE, per min (activity in thyroid in 30 min ALD μM fructose 1 6 phosphate split per min ICDH mM TPNH formed per per min
according to Student s t test P < 0 001 bP < 0 01 P < 0 05

ALD	ICDH	LDH	GOT	GPT
11 3±1 07 (10)	3 6±0 16 (14)	235±13 2 (19)	33 0±2 51 (24)	24 4±1 83 (23)
13 0±0 36 (11)	3 9±0 22 (12)	174±33 0 (24)	35 1±7 71 (17)	26 4±2 65 (16)
101 3±11 16 (10)	1 2±0 13 (15)	275±19 9 (18)	34 4±2 84 (14)	5 1±0 78 (25)
87 1±7 66 (10)b	1 3±0 09 (13)	255±13 8 (24)b	26 3±1 21 (13)	4 2±0 35 (21)
10 6±0 97 (8)	0 9±0 08 (8)	34±4 3 (7)	19 2±2 22 (8)	1 6±0 19 (8)
8 9±1 12 (8)	0 9±0 09 (8)	47±5 9 (8)b	24 0±2 70 (8)	1 8±0 37 (8)
4 6±0 52 (8)	3 3±0 47 (8)	45±3 3 (8)	32 5±3 71 (9)	2 7±0 18 (11)
3 8±0 53 (8)	2 9±0 35 (8)	52±9 6 (7)	18 8±2 84 (8)	1 9±0 19 (10)

TABLE II DNA content in different tissues of intact and hypophysectomized rabbits
Mean ± s e m (number of observations) of the DNA content given in mg per g wet weight Differences between intact and hypophysectomized rabbits have been calculated according to Student t test P < 0 001 bP < 0 01 P < 0 05

	Body wt (kg)	Liver	Muscle	Cerebral cortex	Hypothalamus	Adrenal cortex	Thyroid gland
Intact	3 0±0 20 (6)	3 7±0 37 (6)	0 9±0 05 (6)	7 4±0 10 (6)	5 5±0 37 (6)	5 1±0 60 (6)	7 4±0 40 (6)
Hypophysectomized	2 5±0 13 (6)	6 8±0 45 (6)	1 1±0 0 (6)	2 5±0 1 (6)	5 5±0 45 (6)	1 4±1 69 (6)b	9 0±0 51 (6)

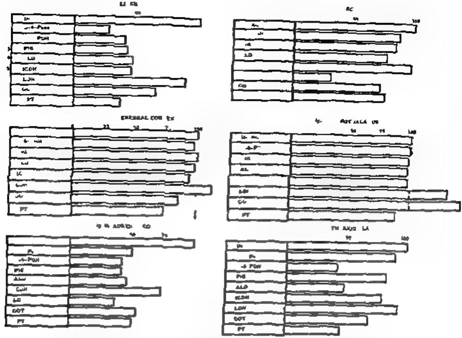

Fig 6—11 Activities observed in hypophysectomized rabbits on the enzymes indicated in Fig 1 The values are calculated per unit DNA For comparison the enzyme activities of intact animals are given as 100 Six intact and six hypophysectomized rabbits were used for each enzyme determination The differences between intact and hypophysectomized animals have been calculated according to Student's t test

< 0 001 Fig 6 1 2 3 4 5 8 Fig 7 6 Fig 10 6
< 0 01 Fig 6 7 Fig 10 1 2 3 4 7 8 Fig 11 4 8
P < 0 05 Fig 7 4 7 8 Fig 9 6 7 Fig 10 5 Fig 11 7

values similar to those obtained from the adrenal cortex (Fig 11) Determinations according to Schmidt et al (1958) resulted in very low values in both intact and hypophysectomized rabbits The causes underlying this peculiar discrepancy of values found in the thyroid are being studied

Phosphohexose isomerase (PHI) and *aldolase* (ALD) Except for liver (PHI) and muscle (ALD) significant changes were not observed in any of the tissues studied when the activities of the enzymes were calculated on the basis of wet weight (Table I) As may be seen from the figures differences appear when the activities are expressed per unit DNA This holds true for all tissues except the cerebral cortex (rats and rabbits) the hypothalamus (rabbits) and muscle (rabbits)

TPN specific iso citric dehydrogenase (ICDH) Subsequent to hypophysectomy the activity of this enzyme calculated per unit DNA was decreased in liver and adrenal cortex ($P < 0.001$) of rats and rabbits and cerebral cortex of rats ($P < 0.05$) Changes did not occur in the other tissues studied (Fig 2—11) When the results were expressed per unit weight no differences could be obtained

Lactic dehydrogenase (LDH) Except for adrenal cortex values expressed per unit wet weight did not reveal any significant changes in the various tissues after hypophysec

tomy The figures obtained for cerebral cortex were higher than in the intact controls (Table I) When the results were based on DNA the activity of LDH was decreased in the liver muscle and adrenal cortex but not in the other tissues studied (Fig 2—11)

Glutamic-oxalacetic (GOT) and *glutamic pyruvic* (GPT) *transaminases* Except for brain a significant decrease in GOT and GPT activities (expressed per unit DNA) was observed in all tissues studied after hypophysectomy (Fig 2—11) In muscle and adrenal cortex of rat but not in the other tissues significant changes were also obtained for GOT when calculated per unit wet weight (Table I)

Glutamic acid dehydrogenase (GlDH) After hypophysectomy the level of glutamic acid dehydrogenase was not significantly changed in liver and muscle irrespectively of how the results were expressed Other tissues were not studied

Water and *fat* content of the liver and muscle were not modified by the removal of the pituitary gland

Discussion

In the present work interest was devoted to amongst other things the calculation of results When the values obtained were expressed per unit wet weight or per unit protein no changes of particularly Embden Meyerhof enzymes could be revealed after hypophysectomy This holds true for the citric acid cycle enzymes and for the transaminases in liver After hypophysectomy a splanchnomicria is generally present and it was shown that the number of cells per weight or volume unit of tissue increases (*cf* Di Stefano and Diermeier 1959) As the DNA content per weight or volume unit of tissue may generally be regarded as indicating the number of cells present expression of results in terms of DNA content would seem more appropriate It should be recalled that in the present work at least two weeks elapsed between hypophysectomy and study of organs The reason for examining the rats and as far as possible rabbits at a set age is the finding that enzyme activities change with age (Ross and Ely 1954b) The results will be discussed in the sequence indicated by Fig 1 The order chosen for organs will be Liver skeletal muscle cerebral cortex hypothalamus adrenal cortex and thyroid gland

1 *Glucose 6-phosphatase (G 6-Pase)*

After hypophysectomy this enzyme necessary for the release into the blood of precursors of glucose-6-phosphate and of free glucose from the glycogen pool was found unchanged in the brain but reduced in liver and muscle (Table I Figs 2—4 and 6—7) The result agrees with that reported by Harper and Young 1959) in liver of hypophysectomized rats Harper and Young (1959) did not find any changes in kidney In this connection it should be mentioned that the procedures used in the present work seemed to permit the detection of G-6-Pase in brain and muscle and that the differences observed in muscle before and after hypophysectomy were significant (Table I Fig 3) The result should be regarded with caution however since the hydrolysis of glucose-6-phosphate in muscle and cerebral cortex might have been catalyzed by phosphatases with low substrate specificity

That the G-6-Pase may be hormone dependent is indicated by the findings of authors who performed studies under other conditions In diabetes for instance elevated levels of G-6-Pase were found in the liver by Ashmore Hastings and Nesbett (1954) and by Langdon and Weakley (1955 Addition of insulin to the alloxan diabetic rats of

Ashmore *et al* (1954) reduced the increased activity Harper and Young (1959) found *that the administration* of growth hormone to hypophysectomized rats resulted in a rise of G 6 Pase level Langdon and Weakley (1955) found that growth hormone or cortisone did not influence G 6 Pase activity in the intact rat Weber *et al* (1956) who used high *doses (25 mg per rat daily)* observed that cortisone caused a marked increase of G 6-Pase activity in intact rats

The decrease of G-6 Pase which occurred after hypophysectomy in the liver appears unlikely *to be due to the absence* of *growth hormone and ACTH TSH* may be more important Rats treated with thyroxine showed a raised G 6 Pase activity in the liver (*cf* Knox *et al* 1956) Tata *et al* (1963) showed that tri iodothyronine produced a marked but transient *stimulation of G 6 Pase in the livermicrosomes of thyroidectomized* rats

Nutritional factors should also be mentioned According to Knox *et al* (1956) a diet low in glucose produced decreased *activities of glycolytic enzymes but not of G 6 Pase* in liver When glucose was replaced by fructose small changes only, were noticed with regard to the activity of G 6-Pase (Fitch Hill and Chaikoff 1959 and Niemeyer 1962)

The decrease in G 6 Pase activity after hypophysectomy was more marked in the rabbit than in the rat In contrast the specific activity wa consid rably higher in the intact rat than in the intact rabbit This is probably due to the fact that rabbits are herbivorous animals which utilize carbon fragments more readily than omnivorous rats

G 6 Pase activities of the adrenal cortex of intact rats and rabbits were low A modification of this situation by hypophysectomy could not be demonstrated As mentioned above when discussing skeletal mu cle and brain tissue the activity found might have been due to unspecific phosphatases

2 Combined activities of glucose 6 phosphate and 6-phosphogluconate dehydrogenases (G 6 PDH)

In the hypophysectomized animals the activity of these dehydrogenases was markedly decreased in the liver and to a lesser extent in muscle It should be mentioned that the activity in muscle of intact animals was very low a finding which agrees with *the results* of Glock and McLean (1955a) The difference found in the brain tissues was negligible

The findings would seem to indicate that the hexose monophosphate pathway is used *to a lesser extent in the liver of hypophy ectomized animals The change should* in part be explained on account of endocrine disturbances Glock and McLean (*1955a*) found that thyroxine treatment produced an increase in the levels of activity of these dehydrogenases *in the liver* In alloxan diabetes decreased values were reported (Glock and McLean 1955b) Tepperman *et al* (1961) reported that restoration of shunt activity occurred when hypophysectomized rats fed a high fructose diet were treated with a combination of growth hormone cortisone and tri iodothyronine With a standard diet tri iodothyronine alone restored the values in the liver of hypophysectomized rats

In the adrenal cortex and the thyroid gland a marked decrease of the combined activities of G 6 PDH occurred after hypophysectomy (with regard to the thyroid *gland* see *Results*) The results support Tepperman and Tepperman (*1960*) *who suggested* that hormone synthesis is *closely related* to the activity of the hexose monophosphate pathway As mentioned above the same pathway is *less active in muscle where glucose* metabolism normally proceeds *almost exclusively via* glycolysis (Glock and McLean 1953)

That the diet may change the activity of the hexose monophosphate enzymes was shown by Fitch *et al* (1959) Of interest is also the finding of seasonal variations in the levels of the two dehydrogenases in the liver (Glock and McLean 1955a) In the present study this observation was confirmed The dehydrogenase concentrations were higher in summer than in winter Seasonal variations were not apparent in the hypophysectomized animals

3 *Phosphohexose isomerase* (*PHI*) and 4 *Aldolase* (*ALD*)

These two enzymes involved in the Embden Meyerhof pathway showed marked similarities after hypophysectomy The results differed between tissues In liver and muscle a decrease in activity of the two enzymes was revealed only when the values were calculated on the basis of DNA No changes appeared in the brain Fitch *et al* (1959) found that the activity of PHI and ALD in rat liver increased when the regular food was replaced by a diet rich in fructose

In the adrenal cortex and the thyroid gland a significant decrease was observed irrespective of the mode of calculation This finding suggests that a decrease occurred not only in the dehydrogenases involved in the pentose shunt but also in enzymes involved in the glycolysis according to the Embden Meyerhof pathway

5 *Iso-citric dehydrogenase* (*ICDH*)

The changes observed after hypophysectomy were practically the same as for LDH (6) The activity found in the various tissues of intact rats and rabbits were not much lower than in the liver ICDH activity in rat liver appears to be relatively insensitive to carbohydrate changes in the diet (Fitch and Chaikoff 1962) Since the authors just mentioned found either an increase (PHI ALD and LDH) or no change (ICDH) the decrease of these enzymes found in the present work can hardly be due to diminished food intake

6 *Lactic dehydrogenase* (*LDH*)

Both in liver and muscle there was a decrease of LDH after hypophysectomy The decrease was significant only when the values were expressed per unit DNA As to liver the activity was low about half that of intact controls There was no difference in the brain

A marked decrease was also found in the adrenal cortex and the thyroid gland although the values observed in controls were considerably lower than in the liver In the study referred to above (Fitch and Chaikoff 1962) LDH was found to increase in a similar fashion as did PHI and ALD

Transaminases (*7 GOT* and *8 GPT*)

In all tissues studied except in the brain the activities of GOT and GPT were decreased after hypophysectomy provided the values were expressed on the basis of the DNA content When calculated per unit wet weight differences were found only with regard to GOT in muscle and adrenal cortex which confirms results obtained by Bartlett and Glynn (1950a and b) on liver and muscle

Rosen *et al* (1959) reported that cortisone or cortisone derivatives increased the GPT activity in liver of intact animals regardless of the basis of comparison Elevated transaminase activities in livers of diabetic animals have been reported by Copenhaver Shipley and Meyer (1951) Rosen *et al* (1959) also showed that in alloxan diabetic rats

the activity of GPT was greatly increased This increase was prevented by administration of insulin Rosen *et al* (1959) further found that the level of this enzyme in liver was directly related to the protein content of the diet Thus the specific activity of the enzyme in the liver of animals fed a high protein diet was increased manifold when compared with control animals fed a diet without protein It should be pointed out that this effect also occurred in adrenalectomized animals Waldorf *et al* (1963) found increases in GOT and GPT in liver of rats which received a limited amount of food or diets rich in casein and fat Feeding low carbohydrate high fat diets caused no change in the activity of either enzyme

9 Glutamic acid dehydrogenase (GlDH)

In the present study the activity of GlDH was changed after hypophysectomy neither in liver nor in muscle It has been reported that the enzyme level may decrease after hypophysectomy (*cf* Knox *et al* 1956) The present study confirms the results obtained by Gaebler and Mathies (see Knox *et al* 1956)

Among the consequences of removal of the pituitary gland is a decreased food intake which in part is due to a lower basal metabolic rate On the other hand hormonal mechanisms exert an indispensable influence under starvation in order to maintain homeostasis (*cf* Knox *et al* 1956) Changes occurring in enzyme activities of animals deprived of their pituitary gland may at least to a certain extent be due to modifications in food intake (Knox *et al* 1956 and Niemeyer 1962) In young and adult rats with intact pituitary gland Ross and Ely (1954a) found that low protein diets resulted within 0 days in a decrease in lactic and succinic dehydrogenases in the liver Waimo *et al* (1959) confirmed the results of Ross and Ely (1954a) and found in addition that food restriction *per se* had little or no effect on the unit activity of the enzymes

Nevertheless in the present work it seemed necessary to begin with a study of enzymatic patterns occurring after hypophysectomy without regard to the quantity of food ingested by the animals Effects of dietary modifications on the enzymes studied in the present work have been mentioned above Further studies on hypophysectomized animals treated with physiological amounts of hormones with known metabolic actions are being performed in order to investigate modifications produced hormonally in the enzyme systems and tissues studied here

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Acta physiol scand 1965 63 285—291

From the Institute of Zoophysiology University of Oslo Blindern Norway

The Importance of the Legs in the Thermoregulation of Birds

By

INGER STEEN and J B STEEN

Received [illegible] June 1964

Abstract

Steen I and J B Steen *The importance of the legs in the thermoregulation of birds* Acta physiol scand 1964 *63* 285—291 — The proportion of the total heat production of herons and gulls which at ambient temperatures ranging from — [illegible] C to 35 C is dissipated to cold water or air surrounding their legs has been measured At low ambient temperatures less than [illegible] of the metabolic heat is lost from the legs At higher ambient temperatures an increasing proportion of the metabolic heat [illegible] lost from the legs and at 35 C almost the entire heat production is dissipated through the legs Heat loss to water was found to be about four times higher than to air of the same temperature The degree of heat loss from the legs was shown to react within seconds to changes in ambient temperature Likewise panting stopped immediately when the legs were irrigated by cold water It is concluded that the naked legs of these birds serve as controlled heat conduits of great importance in thermoregulation

Although the legs of both mammals and birds serve the same main function of locomotion evolution has selected uninsolated legs in most birds but furred legs in most mammals This difference may be related to and may also have influenced the development of different thermoregulatory mechanisms in these two groups of animals Thus it appears as if the naked legs [illegible] birds secondarily serve the same function as sweating does in various groups of mammals Kahl (1963) showed quite convincingly that the legs of the wood stork are important in the thermoregulation of these animals The body temperature of the storks in a hot environment increased when the legs were insulated and decreased again when they were squirted with water Under these conditions they also displayed a unique behaviour they deposited fluid excrements on their legs thus achieving evaporative heat loss as the water was trickling down their legs It may also be significant that the few species of birds which have feathered legs are mostly arctic inhibitants for whom dissipation of large amounts of heat may not be as great a problem as heat conservation.

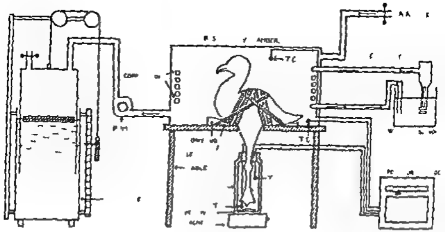

Fig 1 The experimental arrangement used for simultaneous measurements of oxygen uptake and heat loss from the legs

During cold stress birds reduce the heat loss from their legs This is evident from the observation that ducks and gulls do not leave melted foot prints when they sit on ice flakes in the winter Irving and Krog (1954) have actually measured a leg temperature as low as 0 C in a gull at an ambient temperature of — 10 C The finding of Hyrtl (1863) of vascular counter current bundles retia in the tibial region of several longlegged birds indicate one mechanism for heat conservation The tissues of such extremities are apparently adapted to temperatures close to freezing Chatfield Lyman and Irving (1953) thus found that nerve axons in the legs of the herring gull were progressively more cold adapted the further down on the leg they were found A section of the nerve taken from the metatarsal region was able to conduct impulses at temperatures down to 3 C while conduction in a nerve section from the tibial region was blocked at 12 C

The available information thus shows that the unfeathered parts of the bird legs are controllable heat conduits of primary importance The legs are kept cold to reduce heat loss at low ambient temperatures at high ambient temperatures and presumably during muscular activity heat loss from the legs is enhanced

The aim of the present investigation has been to measure that proportion of the heat production of herons and gulls which at ambient temperatures from — 10 to 35 C is given off from the legs to water or to air The rate whereby the legs can change their function from one of heat dissipation to one of heat conservation has also been examined

Material

Three herons *Ardea cinerea* (wt about 800 g) and three gulls *Larus marinus* (wt about 1 000 g) were used in these experiments They were caught as young in July 1962 at Tarva an island a few miles west of Trondheim at the Norwegian west coast In the laboratory they thrived well on a diet of fish and pigs liver The experiments were carried out in January to June 1963

The heron is a migratory bird in Norway whereas this species of gull winters along our coast.

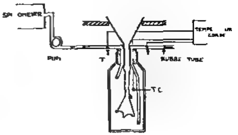

Fig 2 The experimental arrangement used for measuring heat loss from one leg to surrounding circulating air

Experimental

Respiratory chamber

The experimental arrangement shown in Fig 1 The temperature of the respiratory chamber was regulated by placing it in a room of + 5 C or of — 10 C and at the same time leading water of temperatures from 0 to 50 C through the internal copper coil of the chamber Rapid temperature changes were accomplished by suddenly opening the chamber The temperature was measured by thermocouples placed at the roof and floor of the chamber The ambient temperature referred to later is the average of these two temperatures One of two legs penetrated the floor of the chamber and was enclosed in a thermobottle from above the heel

Heat production

Metabolic heat production was obtained indirectly by measuring the oxygen uptake and assuming a calorific value of 4 9 kcal/l O_2 i e an R. Q. of 0 9 Fresh air was sucked through the chamber and pumped into a respirometer where its volume was recorded In this way possible leakage of gas would be directed into the chamber and would thus not affect the measured oxygen consumption which was obtained from the volume of collected gas combined with analysis of the gas composition before and after it had passed through the respiratory chamber The gas analyses were carried out with the method of Scholander (1947)

Heat loss

In order to get directly comparable results we tried to select experimental conditions where the heat loss to water or to air was measured at approximately the same temperature of the two media Due to the great difference between their specific heat however different procedures had to be applied Heat loss from one foot to water was measured by filling a known amount of water at 4 C into the bottle and keeping it stirred with a magnetic stirrer while the increase of temperature was recorded every 20 sec When the temperature reached 12 C the water was changed The temperature was measured by a plastic covered thermocouple held in position in the bottle by a wooden pin inserted in the cork stopper (Fig 1) and recorded on a Honeywell multi channel recorder

Heat loss from the leg to air was measured by sucking a known volume of air of 8 C through the thermos bottle whilst the temperature of the air as it escaped the bottle was recorded (Fig 2) The flow of air was maintained such that the temperature of the outflowing air was kept well below that of the foot itself (18—20 C)

The average air temperature was 13 C in these experiments while the average water temperature was 8 C The effect of the higher air temperature upon the heat loss is partially offset by the fact that air and arterial blood flowed countercurrent to each other in the thermos

Result

The herons could sit absolutely quiet during hours of experimentation with repeated changes in ambient temperature or water temperature The gulls were more restless This difference in behaviour was reflected in the results The data presented are from the most typical experiments on the most cooperative birds of each species

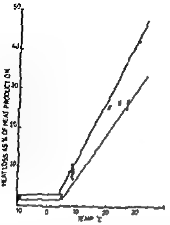

Fig 3

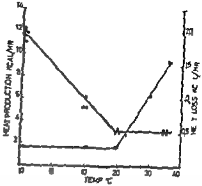

Fig 4

Fig 3 Relationship in gulls between ambient temperature and the relative heat loss from one water immersed leg given as per cent of total heat production The two lines are drawn through the extreme values

Fig 4 Relationship between heat production (●) and heat loss from one water immersed leg (×) of a heron at varying ambient temperatures

Heat production

The average heat production of resting gulls at temperatures between — 10 and 35 C 7 0 kcal/hr (25 determinations ranging from 5 80 to 8 50 kcal/hr) Their critical perature was therefore below — 10 C Above 35 C the birds became restless they panted and their oxygen uptake increased

The average heat production of resting herons at ambient temperature from 20 to 35° C was 3 20 kcal/hr (Seven determinations ranging from 2 9 to 3 4 kcal/hr) At temperatures below 20 C the oxygen uptake increased as shown in Fig 4 Their lower critical temperature under these conditions was therefore about 20 C Above 35 C they showed the usual signs of heat stress

Heat loss

At ambient temperatures below 10 C the gulls lost about 0 2 kcal/hr or less than 3 of their total heat production from one leg bathed in cold water (Fig 3) At temperatures above 10 C the heat loss increased and at 30 C about 40 % of the gull s total heat production was lost from one water immersed foot When the bird was struggling the heat loss from one leg could exceed the resting heat production The maximum heat loss measured from one water immersed foot of a gull was 9 0 kcal/hr or well above the animals resting heat production

Fig 4 gives the relationship between heat production and heat loss from one water immersed foot of a heron Below 20 C the heat loss is about 0 3 kcal/hr At 20° C this is 10 % of the heat production At temperatures above 20 C the heat loss from one water immersed leg increased and at 35 C it was about 60 % of the heat production The maximum heat loss from one foot was 4 2 kcal/hr or somewhat above the resting heat production

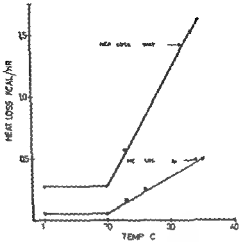

Fig 5 Heat loss from one leg of herons to water and to air at varying ambient temperatures

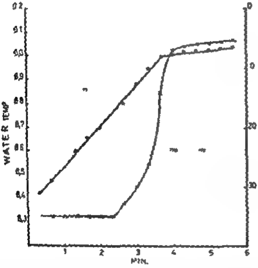

Fig 6

Fig 7

Fig 6 A redrawn recording of the changes in the temperature of the water surrounding one leg of a gull during a rapid change in ambient temperature from 35 C to 5 C.

Fig 7 The vascular rete of the tibial region of the heron Redrawn from Hyrtl (1863)

In Fig 5 the heat loss from one foot of a heron to water and to air is compared Heat loss to air under the conditions of the experiment is about one fourth of the heat loss to water

Rate of change of heat loss

Fig 6 shows the temperature change of the water surrounding one leg of a gull during a rapid change in ambient temperature from 35 C to 5 C. The rise in water tempera

ture changed abruptly within half a minute of altering the ambient temperature The slope of the curve is reduced by a factor of 7 which means that the gull can reduce its heat loss from the water immersed leg by 85 per cent within half a minute Heat loss was further reduced during the following period

Gulls started to pant when they were wrapped in a towel or sometimes when they made vain efforts to get free The panting stopped within a few seconds upon immersing the legs in cold water

Discussion

Homeotherme animals have several mechanisms for heat dissipation in addition to radiant and convectional heat loss from the body surface At high ambient temperatures man relies to a large extent on evaporation of internal water primarily as sweat but also from the respiratory surface In well isolated arctic animals respiratory heat loss plays a more dominant role Hammel (1962) showed that a reindeer may loose 20 % of its heat production from the respiratory tract during strenuous running This amount of heat is about equal to the resting heat production Birds have possibly as a consequence of the requirement for a light body developed mechanisms which are less dependent on internal water King and Farner thus state (1961 p 234) that evaporative heat loss cannot amount to more than 50 % of resting heat production in birds Eliassen (1963) reports that some birds loose only 10 % of their actual heat production from the respiratory tract during flight while a considerable proportion of the heat seems to be lost from the sparsely feathered underside of their wings The present investigation shows that the unfeathered legs of the heron and the gull are able to dissipate a considerable proportion of the heat production An amount of heat equal to or greater than the resting heat production could be eliminated in these birds via one foot immersed in water of 4—12 C About one quarter of the resting heat production could be dissipated from one foot to air in the same temperature range From both legs therefore these birds should be able to dissipate an amount of heat corresponding to twice or half the resting heat production to water or air respectively

Heat loss from the legs to air probably plays a role during flight It has been observed that when herons return from foraging on hot days they wade in water which just covers the bare parts of their legs while fanning themselves with the wings and panting This behaviour is very different from their usual watchful hunting behaviour Heat dissipation in wood storks is augmented by their behaviour of squirting fluid urine on their legs This adaptive behaviour has been termed urohidrosis (urine-sweating) by Kahl (1963)

The changes in heat loss from the legs of the birds investigated is presumably due to variations in blood flow through the leg Technical limitations in our laboratory prevented direct investigation of circulatory changes but we observed that the tibial veins were strikingly more swollen during heat stress than during cold stress

Vascular counter current bundles retia in the lower region of the tibia have been found in some birds among others in herons but not in others like the gull (Hyrtl 1863) (Fig 7) In a rete there will be a net flux of heat and diffusible material between arterial and venous blood according to the direction of the gradient The degree of exchange depends upon the conductivity and diffusivity of the separating tissues as well as on the architecture of the rete A rete with many vessels lying close together will be more efficient than one in which there are few vessels spaced farther apart The degree of exchange will furthermore be greater the slower the blood flows (Scholander and

Krog 1958) The retia of the heron's legs consist of a rather irregular arrangement of a few relatively large arteries and veins It is tempting to suggest that these rather poorly developed retia have a dual function at a low ambient temperature with a small blood flow the heat of the arterial blood is largely transferred to the venous blood while oxygen and carbon dioxide does not exchange appreciably At high ambient temperature with a large blood flow however very little heat transfer occurs between arterial and venous blood In this way the retia will help to conserve heat during cold stress while not affecting heat dissipation during heat stress There is also anatomical evidence for the existence of vascular paths in the legs of these birds whereby blood may be shunted so as to by pass the retia (Fig 7)

The fact that panting can be stopped immediately by squirting cold water on the legs indicates that panting is under some sort of reflex control The observation that heat loss from the leg of a gull may fall sharply to one seventh of its previous value with a deley of less than 30 sec when the ambient temperature is suddenly reduced from 35 to 5 C seems to indicate that the heat loss from the legs is also influenced by reflexes elicited from peripheral receptors Randall (1943) found evidence that heat loss in hens was regulated via the temperature of the hypothalamus The possible relationship between hypothalamic temperature and degree of heat loss from the legs has not been explored in the present investigation but it seems unlikely that these rapid reactions are mediated by the hypothalamic blood temperature

The restricted position of the birds during the present experiments may clearly have disturbed their normal heat dissipation in several ways Conclusions about normal critical temperature and other parameters which depend on the normal functioning of the body insulation can therefore not be drawn The experiments do however demonstrate the potentially great role which the legs may play in the temperature control of these birds

It is a pleasure to acknowledge the late owner of the island Tarva Mr F C Hagen for his enthusiastic assistance in securing experimental animals We are indepted to Prof B Waaler for his thorough revision of the manuscript. The investigation was supported by a grant from Norsk Varekrigsforsikrings Fond.

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Acta physiol scand 1965 63 292—295

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Hyperpotassemia and Electrocardiographic Changes in the Duck during Prolonged Diving

By

HARALD T ANDERSEN

Received 26 June 1964

Abstract

ANDERSEN H T *Hyperpotassemia and electrocardiographic changes in the duck during prolonged diving* Acta physiol scand 1965 63 292—295 — A conspicuous bradycardia and a series of characteristic changes in the electrocardiogram (ECG) may be observed when diving vertebrates submerge The ECG-changes include prolongation of the P—R interval with ultimate disappearance of the P wave and an elevated and peaked T wave These deviations from the normal ECG pattern indicate severe hyperpotassemia It is reported in the present paper that the plasma potassium concentration actually do increase during prolonged underwater exposure The relationship between the ECG changes observed during submersion and the hyperpotassemia is discussed

Bert (1870) discovered that a marked bradycardia is elicited in ducks upon submersion This phenomenon has later been observed in all species of diving vertebrates studied except the porpoises A similar but less pronounced slowing of the heart rate may also take place in non-diving animals and man when exposed to underwater conditions (for review see Andersen 1963 Scholander 1964)

Electrocardiograms (ECG) recorded during diving are characteristically different from those obtained when the animals are breathing air These ECG-changes have been attributed to nervous mechanisms and hypercapnia (Johansen 1959 Murdaugh Seabury and Mitchell 1961) However the progress of the electrocardiographic changes during diving are also very similar to those observed during hyperpotassemia Increasingly prolonged P—R interval with ultimate disappearance of the P wave and elevated and peaked T wave (Winkler Hoff and Smith 1940) Depression of the S—T segment may also be seen but this parameter is rate-dependent (Bazett 1920) and is therefore difficult to evaluate during prolonged submersions

Since the ECG-changes during diving are suggestive of elevated plasma potassium a study was undertaken in order to determine whether prolonged underwater exposure really causes hyperpotassemia The results are reported in this paper

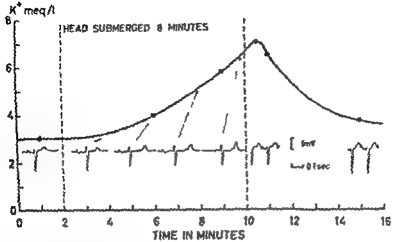

Fig 1 ECG (lead III) and concentration of K ions in blood plasma before during and after a dive Arrows indicate plasma potassium level corresponding to each ECG-cycle recorded during period of submersion

Methods

Eleven domestic ducks 1—2 years old were studied Their body weights ranged from 2 0 to 3 0 kg Conditions of diving were simulated as described previously (Andersen 1959 1961 1963)

Needle electrodes were inserted subcutaneously into the thighs and at the base of the wings and the ECG was recorded using an Elema-Schonander Mingograf Model 42 A wing artery was cannulated and blood samples 1 ml each were withdrawn at suitable intervals during the dive The blood was centrifuged at 2 200 rpm for 10 min. The concentration of potassium in plasma was determined by means of an Evans Electroselenium Flame Photometer Mod 1 A

Results

The results obtained in an experiment in which the duck endured 8 min of submersion are shown in Fig 1 The plasma of the resting bird contained 3 0 meq l of potassium ions This value was doubled in less than 7 min of submersion The increase in plasma potassium persisted through the first minute in the recovery period simultaneously with a continuous drop in pH (Andersen Hustvedt and Løvø 1964)

Also shown in Fig 1 are ECG tracings recorded at various stages during the under water exposure It appears that the P wave soon became diphasic and that the P—R interval was prolonged from 60 to 110 msec until finally the P wave entirely disappeared The amplitude of the T wave increased from 0 20 to 0 60 mV and became distinctly peaked in appearance Detailed information on the degree of hyperpotassemia and the corresponding changes in the ECG is provided in Table I

Results very similar to those reported in Fig 1 and Table I were obtained in every one of 15 expts

TABLE I. ECG-changes (lead III) related to concentration of K^+ in blood plasma of duck during diving experiment

K^+ meq/l	Appearance of P wave	Amplitude of T wave mV	Duration of P R interval msec	Remarks
3.0	Present, monophasic	0.20	60	Before dive
3.3	Present, diphasic	0.30	90	2 min submerged
4.0	Present, diphasic	0.45	90	4 min submerged
5.2	Present, diphasic	0.50	110	6 min submerged
6.7	Absent	0.60	—	8 min submerged
7.0	Present, monophasic	0.30	55	After dive 1 min
3.7	Present, monophasic	0.30	55	After dive 5 min

Discussion

A marked hyperpotassemia develops during prolonged underwater exposure and the ECG simultaneously recorded shows the changes typical of this condition suggesting that the electrocardiographic changes are not caused by nervous influence and accumulation of carbon dioxide only, but that the hyperpotassemia in part is responsible for the deviations from the normal ECG pattern.

The hyperpotassemia can not be the only cause of the electrocardiographic changes, however, since the latter do not persist during the early part of the recovery period when the hyperpotassemia is maximally developed. The T wave certainly remains elevated, but the P wave reappears and the P—R interval attains its normal duration. On the other hand, upon emersion the heart accelerates from approximately 20 to 400 beats/min (Andersen 1963), which leaves no time for prolongation of any ECG interval. Besides, carbon dioxide is rapidly blown off and the nervous inhibition of the heart via the vagi is also abolished, so that the effects of the hyperpotassemia may be largely suppressed. That the latter influence the ECG during the dive is, however, clearly indicated by the results here presented.

This study was supported by a grant from Nansen Fondet.

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Acta physiol scand 1965 63 296—310

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The Pattern of Muscular Activity During the Arm Swing of Natural Walking

By

Maria L Fernandez Ballesteros[1] Fritz Buchthal and Poul Rosenfalck

Received 26 June 1964

Abstract

Ballesteros, M L F F Buchthal and P Rosenfalck *The pattern of muscular activity during the arm swing of natural walking* Acta physiol scand 1965 63 296—310 — *A telemetering device is* described to record action potentials from muscles of the upper arm and the shoulder during the arm swing of natural walking The forward swing is caused by activity in some inward rotators the flexors remaining inactive The posterior part of the deltoid and some outward *rotators are responsible for the backward swing During walk turning electrical activity occurred in flexors inactive during straight walking accounting for the increased excursion of the forward swing which counteracts the torsional movement of the trunk Step-related muscular activity* persisted even when the arm was prevented from swinging

Involuntary swinging of the arms during walking serves to counteract rotation of the trunk This function would be accomplished as well if the arms swung passively as if they were moved actively In the early studies of human gait swinging of the arms was described as a passive pendular movement (Gerdy 1829 Weber and Weber 1836) an idea which is still encountered in textbooks on locomotion (Morton and Fuller 1952) Du Bois Reymond (1909) on the other hand conceived arm swing as a passive and an active movement a view supported by Elftman's (1939) analysis of the angular momentum and of the acceleration of the arms In view of the force required to account for *these arm movements* Elftman (1939) concluded that the arm is made to swing largely by muscular action pendular action due to gravity playing a minor role In this analysis however no distinction was *possible between force exerted by muscular* contraction and by *passive elasticity shortening of* a muscle against a load and passive release of a stretched muscle tendon or ligament would both appear as muscle torques in Elftman's analysis

Telemetering *of action potentials* allows the study *of muscle activity* during natural walking we have used the method to record the electromyogram of the muscles of the shoulder and upper arm during walking to establish which muscles are active and to what extent and *when the arm is moved by muscular contraction*

[1] Working under a W H O fellowship

Method

Material The normal subjects were 21 males and 2 females 16 were 18 to 30 years old 4 were 30 to 40 years old 2 were above 50 and 1 was 16 years of age. There was neither sex nor age difference in the pattern of electrical activity during walking In addition findings in two patients are included since they elucidated the mechanism of arm swing during walking an 18 year-old man incurred complete disruption of the axillary nerve during dislocation of the upper head of the humerus a 19-year old man suffered a nearly complete lesion of the upper part of the brachial plexus in a traffic accident

Experimental situation The subjects and patients were instructed to walk with natural posture stride and speed along a corridor 2 5 m wide and 16 m long The electromyogram was recorded from the muscles of the shoulder and upper arm during walking ahead during walk turning and at the onset and cessation of locomotion To familiarize the subject with the experimental situation and to ensure that he walked naturally he was asked to walk up and down for several minutes before he was observed and before recording was started In addition activity was recorded from four subjects seated and standing who were asked to allow their arm to swing freely back and forth in a pendular fashion and to different degrees and from five subjects where arm swing was prevented by loosely binding the arm to the trunk

Measurement of the relation between arm and leg movements during walking To correlate swinging of the arm with the position of the leg a contact was attached to the heel ipsilateral to the arm examined sometimes to the contralateral heel as well Closure of the contact marked the moment when the heel struck the ground

Between two contacts of the ipsilateral heel with the floor the arm swings from backward to forward and back again To correlate the position of the arm with the signal marking contact of the heel with the ground and to determine the time required for a cycle of the arm movements motion pictures (32 frames/sec) were taken in profile of three normal subjects walking on a tread mill at a speed of 50 steps/min The tread mill moved 70 cm/sec A lamp was attached to the subject's wrist the flash of this lamp initiated by closure of the heel contact and points marked on the shoulder and on the foot were used as targets for measuring The single frames were magnified 75 times and the duration of the phases of the movements was determined from the number of frames required for their completion

Leading-off electrodes Needle electrodes were used usually concentric electrodes with an external diameter of 0 45 mm a platinum core 150μ in diameter and a leading-off area of 0 07 mm The impedance of the electrode was 180 kΩ/75° at 50 cps 30 kΩ/6° at 500 cps and 7 kΩ/30° at 5 000 cps Shortly after insertion they caused little or no inconvenience and artefacts due to movement of the needle were rare

Placement of the electrode After insertion the needle was moved slowly until a position was found where brisk activity was recorded during voluntary flexion or extension without resistance and against resistance In muscles which do not flex or extend the arm at the shoulder the electrode was placed where an interference pattern was recorded during abduction adduction rotation or elevation of the arm That the activity recorded from a point was representative for that muscle was ascertained in preliminary experiments by recording simultaneously from three different regions in the same transverse plane of the muscle and by recording simultaneously with a concentric electrode and between two platinum wires placed 10—20 mm apart in the muscle The wires 100μ in diameter and insulated except at the tips (leading-off area 0 9 mm) were inserted into the muscle through a cannula which was then withdrawn Apart from a slightly higher amplitude with these wire electrodes there was no difference in the degree of activity recorded with the two types of electrode

Radio-telemetering To allow free movement of the subjects and patients unrestrained by wire connections between the electrodes and the recording instrument the electrical activity of the muscles and the signal of the heel contact were transmitted by radiotelemetering

In recent years a number of circuits for the telemetering of electrophysiological data have been reported (for references see Kamp and Storm van Leeuwen 1961 Fischler and Frei 1963 Ko Thompson and Yon 1963 Moore Ferrand and Thornton 1963) Snee Gotze *et al* (1958) described telemetering of muscle action potentials by portable vacuum tube circuits the weight and size of the transmitters have been reduced and amplifier properties have been improved In a study of the function of leg muscles in locomotion Abe *et al* (1958) described radio telemetering of the electrical activity of muscles simultaneously

The transmitter we used for multi-channel recording weighed 50 g including batteries and a calibrator which sent a signal of known voltage at short intervals Four transmitters were

placed on the head of the subject (Fig 2) their weight did not cause inconvenience

Each transmitter (size 4 × 6 × 2 cm) consisted of a reactance modulated oscillator with single sided input The modulating voltage was fed from an emitter follower via a condenser to the oscillator The oscillating frequencies were between 100 and 108 Mc/sec a frequency range in which there is no broadcasting station in Scandinavia The transistors in the input and in the oscillator were selected for low noise at the operating points Vibration of the air wound oscillator coil (length 14 mm diameter 12 mm) was prevented by melting polyethylen between the windings Each of the 4 transmitters had a separate current supply to avoid pick up from the leading-off electrodes connected to the other transmitters The current drain was 0 6 mA To telemeter closure of the heel contact one of the transmitters contained the circuit shown lower left in Fig 1

The receiver had 4 channels carefully screened from each other Each channel contained a tuner a 3-stage intermediate frequency amplifier and a radio detector The tuner and the amplifier are parts of commercially available frequency modulation radio equipment

The 4 intermediate frequency amplifiers were tuned to 10 1 10 4 10 7 and 11 0 Mc/sec respectively To counteract frequency drift of the transmitters due to changes in temperature or other causes the receivers were provided with an automatic frequency follower made especially efficient by connecting it via a difference amplifier to a varicap diode In preliminary experiments the transmitters were placed close to the leading-off electrode but transmission was unsatisfactory when the subject turned while walking and the body came between the transmitting and the receiving antennae Therefore the transmitters were placed on the head of the subject (Fig 2) This placement of the transmitters necessitated electrode cables about 100 cm long Drift in oscillating frequency of the transmitter due to movements of the electrode cable was prevented by inserting high frequency chokes between the terminals of the electrode cable and the input of the transmitter

The frequency response was determined with short-circuited input and with a concentric electrode without and with a 100 cm cable (Fig 3) The electrode was placed in 0 15 per cent sodium chloride solution which has an impedance similar to that of muscle With the electrode connected to the input both directly and via the cable the frequency response was 6 db down at 20 cps The upper limiting frequency was determined by the electromyograph the frequency response was 3 db down at 10 kc/sec (Fig 3) The frequency response reflects the fact that the electrode impedance at low frequencies is of the same order as the input impedance and there is therefore considerable distortion of slow initial and terminal components of the single motor unit potentuals On the other hand the pattern of activity was recorded without distortion (Fig 4)

An input signal of 3 mV peak to peak caused a full deflection of the F M detector i e a change in frequency of 100 kc/sec. The input impedance was 100kΩ 20 – 30 μμF The equivalent input noise of the transmitter and receiver was 0 5 – 1 0 μV peak to peak with short circuited input and 3 μV r m s with a concentric electrode in 0 15 per cent sodium chloride connected to the input

Thus the signal to noise ratio received was 70 db with short circuited input and 60 db with a concentric electrode connected to the input The signal to noise ratio was constant over a distance of 7 m with the antenna of the receiver placed vertically This antenna was a common dipole antenna of 70 Ω Deviation from linearity was less than 2 per cent for input signals of up to 10 mV The dynamic range of the input was 60 db (from 3 μV to 3 mV) A continual check of the sensitivity of the transmitter and the receiver was obtained by applying a 1 msec rectangular pulse to the oscillator resulting in a signal which corresponded to an input signal of 100 μV ± 10 per cent from a concentric electrode (Fig 1) Each of the four transmitters emitted its own calibration signal at slightly different intervals (about 1 per sec) This facilitated identification of transmitters during the tuning of the receivers

The calibration signals helped to avoid the following source of artefact when several transmitters were used simultaneously the second harmonic of the oscillator frequency of one transmitter may mix with the basic frequency of another transmitter and thus give rise to simultaneous pick up of signals from 2 transmitters by one receiver For example a basic frequency of 103 Mc/sec in one transmitter could mix with a second harmonic of 210 Mc/sec in another transmitter to emit a frequency of 107 Mc/sec Since this frequency contained the calibration signals of 2 transmitters at slightly different frequencies it could easily be identified as artefact

Recording instrument The receiver was connected to a 3 channel DISA electromyograph (Type 13A69) and the pattern of electrical activity was recorded on photographic paper moving at 5 cm/sec For each muscle recordings were taken during 10 strides Simultaneous

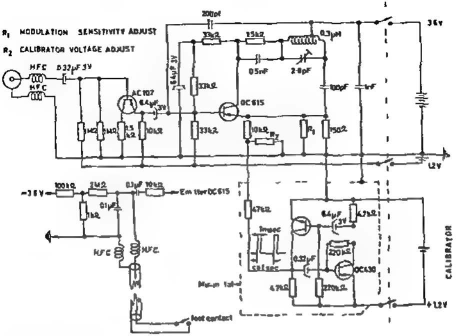

Fig 1 Diagram of the transmitter (above) the calibrator (below to the right) and of the connections to indicate closure of the heel contact (below to the left)

Fig 2

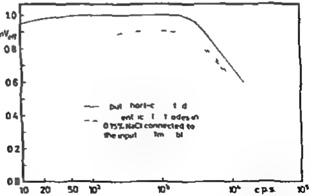

Fig 3

Fig 2 Four transmitters (a) placed on the head of a subject concentric electrodes in the deltoid muscle (b) cable to the foot contact (c) and contact attached below the heel (d)

Fig 3 Frequency response of the transmitter receiver and recording device with short circuited input and with a concentric electrode connected to the input of the transmitter To simulate the impedance of muscle the electrode was immersed in 0 15 per cent sodium chloride solution

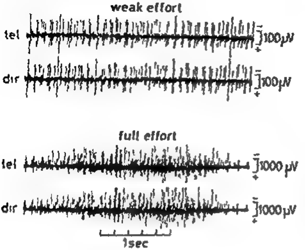

Fig 4 Pattern of electrical activity in the brachial biceps recorded simultaneously by telemetry (tel) and by cable (dir) during weak and during full voluntary effort

recording on an ink writer gave a rough impression of the pattern of activity in the different muscles during the experiment

In 4 subjects the mean voltage of the electrical activity during walking was recorded by an averaging device (Rosenfalck 1959) and compared with the mean voltage of the muscle during full voluntary effort

Results

The relation between the phases of the arm swing and the leg movements

The step length from take off to landing of one foot (a double step) averaged 1 5 m the velocity of walking corresponding to 80 single steps/min (a single step reckoned between successive contacts of right and left heels with ground) Measured from the profile motion pictures (32 frames/sec) of 3 subjects walking 50 double steps/min on the treadmill the duration of an arm movement from backward to forward was 0 57 sec, S D 0 03 sec and from forward to backward 0 57 sec S D 0 015 sec (11 measurements) Maximum backward swinging was simultaneous within 0 03 sec with contact between the heel and the ground The arm remained in the position of maximum backward or forward excursion for at most 2 frames (0 03 sec) The contralateral heel struck the ground exactly midway between 2 contacts of the ipsilateral heel with the ground The ipsilateral foot left the ground at one fourth (0 37 sec S D 0 03 sec) of the double step duration the time when the arm swung past the trunk

Measured from the position of the targets on the wrist and shoulder the angle of movement of the arm from backwards to forward was 30 S D 2 To determine the extreme angle of forward and of backward swing it might seem reasonable to use a point on the trunk as reference This proved impracticable because the trunk rotates during walking and because a target on the trunk is hidden intermittently by the arm Therefore the maximum backward and forward position of the arm was characterized by the maximum angle between the vertical through the target on the shoulder and the line between the targets on the shoulder and the wrist With the arm in the maximum forward position the angle was 20 S D 1 7 and with the arm in the maximum backward position it was 9 S D 1 8 i e measured in this way the angle with the arm swung forward was twice as great as the angle with the arm swung backwards

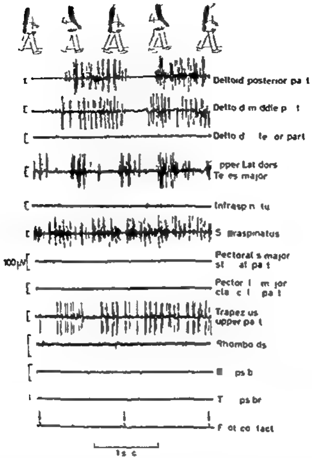

Fig 5 Example of the pattern of electrical activ ty in the muscles of th right shoulder and the upper arm dur g walking Subject 19 male ■ years old length of the double step 1 5 m duration 1 5 sec Above pos tion of the arms as determ ned by cinematography The short pulses at one second intervals are em tted by the calib tor in each transmitter nd h ve an amplitud of 100 µV The s gnals marked by the rrow on th bottom trac nd cate the cl sure of a contact when the r ght heel touched the ground

Since the time taken to swing the arm backward and forward was the same and since the heel contacted the floor simultaneously with the maximum backward excursion of the arm the arm s maximum forward excursion occurred just midway between 2 ipsilateral heel contacts Considering then a double step cycle as the time betw een two ipsilateral heel contacts mechanical activity in the first half of each cycle was related to forward swinging of the arm and in the second half to backward swinging This was confirmed in the experiments in which a foot contact was attached to both heels Finally when relating the electrical activity to a movement regard must be given to the gradual development of the mechanical response (time to peak 50—100 msec Merton 1954) Thus electrical activity which appeared in the last 100 msec of the for

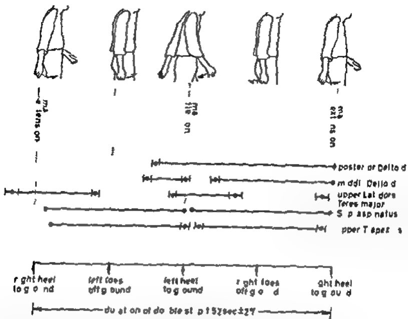

Fig 5 Periods of electrical activity in muscles of the shoulder and upper arm during walking as related to the different phases of the arm swing and the leg movements
Above position of the arm as determined by cinematography
The uncertainty (mean error) of the duration of activity and of the step-dependent silent periods are indicated by the vertical lines or by the diameter of the symbols

ward movement (posterior part of the deltoid Fig 5 6) not only corresponds to the force arresting the forward swing but also initiates the backward movement

Electromyography of the muscles of the shoulder and upper arm during walking

Swinging of the arm from forward to backward can presumably be effected by extensors and outward rotators of the arm at the shoulder listed in Table I

Electromyography (an example is shown in Fig 5) demonstrated activity in 2 of the 3 prime extensors (upper part of the latissimus dorsi and teres major) in one of the assistant extensors (posterior part of the deltoid which also assists in outward rotation) and in the middle portion of the deltoid (an abductor) Abduction allows the arm to clear the trunk in both backward and forward swings Activity was absent in the other muscles which act as extensors or external rotators of the arm at the shoulder (the sternal part of the pectoralis major long head of the brachial triceps teres minor coracobrachial and generally the infraspinate muscles) Activity was also absent in the lower two thirds of the latissimus dorsi

The total duration of arm swing from forward to backwards was 760 msec SD = 50 msec The sequence and duration of electrical activity in the different muscles during swinging of the arm from forward to backward was as follows (Fig 6) activity started in the posterior part of the deltoid 160 ± 13 msec before the arm had attained its maximum forward swing and was nearly simultaneous with activity in the middle portion of the deltoid Briefly thereafter (100 msec) activity appeared in the teres major and upper part of the latissimus dorsi muscles and ceased when the arm reached the vertical position Activity persisted in the posterior and middle parts of the deltoid

TABLE I Prime movers (P M) and Assistant mo ers (Asst) of the upper arm in the shoulder joint[1]

Muscles	Extension	Outward rotation	Flexion	Inward rotation	Abduction
Teres major	P M [2]			P M [2]	
Latissimus dorsi upper part	P M [2]			Asst [2]	
Pectoralis major sternal part	P M			Asst	
Posterior part of deltoid	Asst	Asst			
Triceps long head	Asst				
Infraspinate		P M			
Teres minor		P M			
Coracobrachialis		Asst	Asst	Asst	
Subscapularis				P M	
Middle part of deltoid					P M [2]
Supraspinate					P M [2]
Biceps long head					Asst
Anterior part of deltoid			P M	Asst	Asst
Pectoralis major clavic part			P M	Asst	

[1] Selected from Rasch and Burke (1963)

[2] Muscles found in this study to be active during the arm swing of natural walking

precisely until the final posterior position was reached In 12 of the 23 subjects the activity of the middle part of the deltoid ceased briefly (160 ± 2 msec) just when the arm had attained the final forward position The major role of the posterior deltoid for backward swinging of the arm during walking is shown clearly by the findings in a patient whose axillary nerve had been severed by dislocation of the head of the humerus one month previously (Fig 7) At the time of investigation there was no pain edema nor sign of involvement of other nerves Electrical activity other than denervation potentials was absent in the three portions of the deltoid during full voluntary effort and during walking The motion picture showed the excursion of the arm from forward to backward to be limited the arm no longer passing posterior to the trunk The activity in the supraspinate muscle was five times that found in normals the muscle probably substituting for the middle portion of the deltoid Similarly the activity in the upper part of the trapezius muscle exceeded that in normals

Swinging of the arm from backward to forward can presumably be effected by the flexors and internal rotators of the arm at the shoulder listed in Table I

Electromyographs an example is shown in Fig 5) demonstrated no electrical activity during normal walking in the flexors of the arm at the shoulder nor in other assistant internal rotators than the upper part of the latissimus dorsi Of the 2 primary internal rotators teres major exhibited electrical activity simultaneously with the upper part of the latissimus dorsi Recording from the other primary internal rotator the subscapular muscle was attempted in 6 subjects in 3 the electrical activity was simultaneous with that in the teres major muscle and in 3 it was difficult to ascertain whether the electrodes had remained in the subscapular muscle Electrical activity in teres major the upper part of the latissimus dorsi (and in the subscapular muscle in

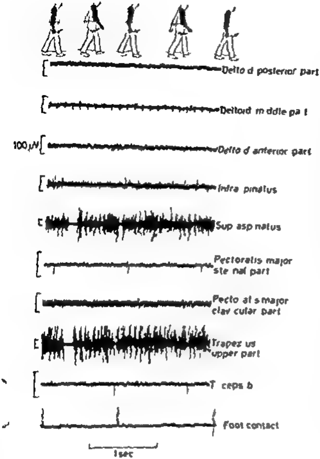

Fig 7 Pattern of electrical activity in the muscles of the right shoulder and the upper arm from a patient with paralysis of the right axillary nerve (patient K A K J male 18 years old)

Above position of the arm as determined by cinematography The arm swing from forward to backward was reduced the arm no longer by passing the trunk The mean voltage of the activity in the abductor muscles and in the upper part of the trapezius muscle was five times that in normals compensating for the paralysis of the middle part of the deltoid muscle The discharges recorded in the deltoid muscle were positive denervation potentials

The signals marked by the arrows on the bottom trace indicate the closure of a contact when the left heel touched the ground

the three successful recordings) started slightly before (150 msec) the arm attained its final posterior position and continued until the arm swung forward to the vertical position (Fig 6) (The similar activity of these muscles during arm swing from forward to backward is described in the preceding section)

Swinging of the arm both backward and forward freely requires abduction which can be effected by the middle part of the deltoid and by the supraspinate muscles assisted by the long head of the brachial biceps and by the anterior part of the deltoid muscles (see Table I) In fact there was continuous electrical activity in the supraspinate and in the upper part of the trapezius (an elevator of the shoulders) muscles except for a brief silent period when the arm changed direction from forward to backward (90 ± 13 msec) and from backward to forward (150 ± 22 msec) In subjects who walked with a stoop (skier's gait) electrical activity was recorded in the rhomboid muscle (an adductor of the shoulder 8 of 19 subjects) and in the infraspinate muscle (9 of 23 subjects both during the forward and backward swing (Fig 8) When present the activity was slight was not grouped according to the phase of swing but ceased briefly when a heel struck the ground (Fig 8) i e at the moment when the arm was swung maximally anteriorly or posteriorly That the electrical activity recorded during

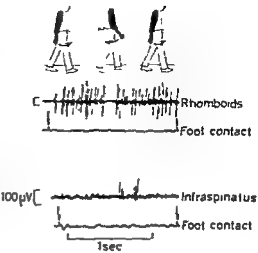

Fig 8 Example of the electrical activity during the arm swing in the rhomboid (in 8 of 19 subjects) and the infraspinate muscles (in 9 of 23 subjects) in subjects who walked with a stoop Above position of the arms as determined by cinematography
The signals marked by the arrows on the bottom traces indicate the closure of the foot contact when the heel touched the ground

arm swing was in fact associated with movements of the arm during walking was evidenced by its immediate cessation when walking was arrested

The degree of electrical activity in the muscles of the shoulder and upper arm during walking was slight as compared to the activity recorded during full voluntary effort against resistance In the extensors the mean amplitude of the electrical activity averaged 10 per cent of the mean amplitude at full effort 4 per cent in the infraspinate muscle 10 per cent in the internal rotators and 15 per cent in the abductors

In a patient with an upper type of brachial plexus palsy (Duchenne Erb) with severe loss of motor units in all parts of the deltoid muscle electrical activity in the posterior and middle portions of the deltoid muscle was of the same degree when the arm swung from backward to forward as during full voluntary effort indicating activation of all surviving motor units

The pattern of activity in free pendular movements with the subject standing As long as the movement was within 20—30 activity was absent in the flexors and in the internal rotators during the swing from backward to forward When the arm swung from forward to backward activity was confined to the posterior part of the deltoid the other extensors being inactive (Fig 9a Hence the forward swing was caused solely by passive forces When the total excursion of the arm exceeded 30 a pendular excursion greater than that in walking activity occurred in a number of other muscles (Fig 9b) Whether or not there was activity in the abductors and in the anterior part of the deltoid depended on the position of the trunk if the arm could swing freely past the trunk abductors remained inactive

To investigate whether muscular activity was the primary phenomenon or was initiated by the pendular movement during walking action potentials were recorded in five subjects whose forearms were loosely bound to the trunk preventing arm swing Fig 10 Under these conditions electrical activity occurred in the upper part of the latissimus dorsi and the teres major and slight activity in the posterior part of the deltoid muscle The activity appeared related to the step as during unrestrained walking Similarly there was activity in these muscles when the subject rotated the trunk in a standing position whether the arm was free or restrained In two subjects where the

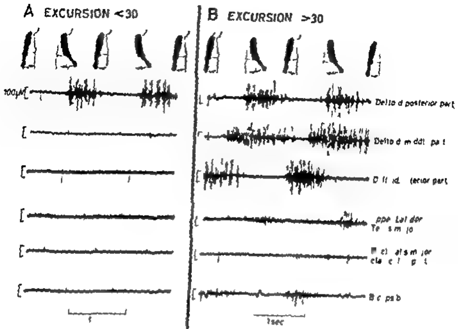

Fig 9
A) Pattern of electrical activity in the muscles of the right shoulder and the upper arm during pendular movements with the subject in a standing position (Subject 3) 22 years old) The arc of the pendular movement was less than 30 as in the arm swing during walking Activity was confined to the posterior part of the deltoid during the swing from forward to backward
B) Pendular movements exceeding 30

rhomboids were examined there was step related activity during walking with the arms fixed Activity was absent in the middle part of the deltoid muscle in four of the five subjects All arm muscles remained inactive if a subject with the arms unrestrained was asked to walk without swinging of the arms

To investigate at what stage in life arm swing developed 53 children 1—5 years old were observed during walking by Dr Melchior[1] At the age of 3 to 5 years when the walking pattern was well established arm swing was present as in adults At the age of 2 one half of the observed 16 children had arm swing during walking and in only ? of 12 one year old children was there a tendency to swinging of the arms

Reversing the direction of walking briskly made the arm swing forward to a greater degree than during straight walking and electrical activity appeared in muscles which otherwise were silent (Fig 11) Thus in all subjects electrical activity appeared in the rhomboid contralateral to the side of turning when the arm swung forward probably antagonizing the simultaneous activity in some of the flexors of the shoulder joint such as the short head of the brachial biceps the clavicular part of the pectoralis major and in 6 of 23 subjects the anterior part of the deltoid muscles Furthermore when the arm was in its maximum posterior position activity started in teres major and the upper part of latissimus dorsi and lasted longer than during straight walking for about two

[1] Pediatric department University Hospital Copenhagen

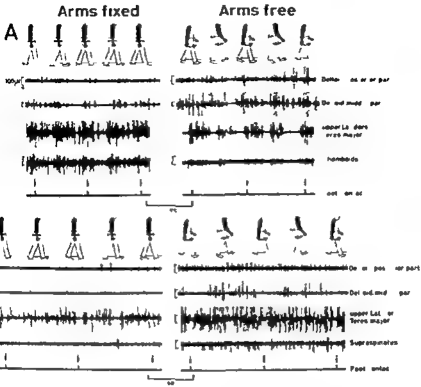

Fig 10 Pattern of electrical activity in the muscles of the shoulder and upper arm during walking with the arms loosely tied to the trunk (left) and during natural walking (right)
A subject P P male 21 years old
B subject H P P male 20 years old
The signals marked by the arrows on the bottom traces indicate the closure of a contact when the left heel touched the ground

thirds of the turning movement. The activity in the posterior and middle portions of the deltoid muscle differed in different subjects in 10 it was as during straight walking in 5 it was of shorter duration and occurred as an 80 msec burst at the end of turning and in 3 these portions of the deltoid muscle were silent The mean amplitude of the activity during turning was the same as during straight walking except for the teres major and the upper part of latissimus dorsi where the amplitude doubled during turning In muscles active solely during turning the mean amplitude was 0 5 per cent of that during full effort in the brachial biceps 3 per cent in the anterior deltoid and █ per cent in the rhomboid

Discussion

From an analysis of Braune and Fischer s (1895) three-dimensional recordings of trunk and limb movements during walking Elftman (1939) confirmed the concept of Gerdy (1829) and of Weber and Weber (1836) that the swinging of the arms counteracts the rotation of the trunk induced by the swinging of the leg The angular momentum of

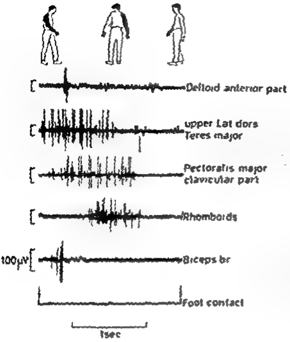

Fig 11 Pattern of electrical activity in the muscles of the right shoulder and the upper arm when the subject reversed the direction of walking by turning to the left (Subject 30 26 years old)
Above position of the arm as determined by cinematography
The signals marked by the arrows on the bottom trace indicate the closure of a contact when the right heel touched the ground
Note the discharges in flexor muscles inactive during straight walking (cf Fig 5) The activity in the upper part of the latissimus dorsi and the teres major lasted longer than during straight walking

the arms around the vertical axis was of the same size but of opposite sign as that of the trunk Elftman (1939) calculated the torques acting on the arms in the different phases of the step He interpreted his findings as indicating the presence of muscular activity However the torques which he calculated could also be due to elastic forces and no attempt was made to distinguish these passive from true active forces At any rate his results did not confirm the concept of Weber and Weber (1836) that gravity can account for the arm swing

In terms of muscular activity the calculated torques would correspond to abduction during both phases of the arm swing to flexion and inward rotation during the swing forward and to extension and outward rotation during the swing backward Thus gravity passive elastic forces and muscular forces act in parallel to produce the arm swing

There was in fact electrical activity in the abductors during both phases of the arm swing enabling the arm to pass by the trunk However the recording of the electrical activity showed that the movement from backward to forward was brought about by some of the inward rotators rather than by the prime or assistant flexors The movement from forward to backward on the other hand was associated with activity of some of the prime and assistant movers of extension the posterior deltoid the latissimus dorsi and the teres major muscles These latter also acted as inward rotators during the swing from backward to forward The fact that step-related electrical activity was recorded even when the swinging movement was prevented indicates that the innervation of the muscles during the arm swing is part of a centrally determined pattern of locomotion This probably includes inhibitory impulses to certain muscles since none of the extensors normally silent could replace the posterior deltoid when it was paralyzed

Furthermore the disturbed pattern of innervation in patients with the Parkinsonian syndrome which could not be explained either on the basis of rigidity or of tremor points to a centrally determined pattern of locomotion (Buchthal and Fernandez Ballesteros 1964)

The role of proprioceptors in this pattern of locomotion has been object of a number of studies in animals Graham Brown (1912) believed to have evidence for a locomotory centre within the brain which could maintain rhythmical limb movements even after complete deafferentation Gray (1956) states that intactness of a single dorsal root no matter at which level is necessary and is sufficient to allow stepping movements to be elicited On the other hand Ten Cate (1962) found locomotory movements of the hind limbs only in response to stimulation of their proprioceptors or exteroceptors In an electromyographic study of chronically deafferented hind legs Wiesendanger (1964) found stepping patterns to occur though at times disturbed by co-contractions of agonist and protagonists However whether these results from animal experiments can be applied to interpret the mechanism of the arm swing seems rather doubtful Although the arm swing is often considered analogous to the movement of the forelimbs in four footed animals (Gray 1956) the function of the muscles of the shoulder and upper arm is to counteract the rotation of the body induced by the swinging of the legs The following observations are consistent with the concept that arm swing during natural walking and the fixation of the shoulders when arm swing was prevented are two different aspects of this act of balancing Elftman's finding (1939) that the angular momenta of trunk and arms were of the same size but of opposite sign and our findings that there was activity in the posterior deltoid muscle when the trunk was rotated in a standing position and an increase in extensor activity during walk turning Thus arm swing a movement of co-operation rather than an automatic movement (Wilson 1929) seems essentially different from the stepping of the forelegs in four footed animals

The trapezius and the rhomboid muscles are active during both phases of the step The activity ceased as soon as the subject stopped walking and stood at ease This is at variance with the low grade postural activity in the upper part of the trapezius during sitting or standing described by Wachholder and Altenburger (1925) Inman et al 1944 and by Basmajian (1967)

Summary

Cinematographic recording of the arm swing during natural walking showed that the forward swing was 20 and the backward swing 9 Action potentials from muscles of the shoulder and upper arm transmitted by telemetry occurred in some inward rotators (teres major upper part of latissimus dorsi and subscapularis muscles during the forward swing and in some extensors and outward rotators posterior part of deltoid teres major and upper part of the latissimus dorsi muscles during the backward swing Neither prime nor assistant flexors were activated during the forward movement. In addition there was activity during both phases of arm swing in abductors (middle part of deltoid and supraspinatus muscles allowing the arm to pass by the trunk and in muscles which fix the scapula to the trunk trapezius and rhomboid muscles)

The mean amplitude of the action potentials in muscles acting during the arm swing was 5—10 per cent of the activity during full voluntary effort

During free pendular movements activity was confined to the posterior deltoid as long as the angle of movement was less than 30

The backward swing was abolished when the posterior deltoid was paralyzed and the brachial triceps normally inactive was not activated to compensate for the absence of activity in the posterior part of the deltoid

Step-related activity appeared in the upper part of the latissimus dorsi and the teres major even when arm swing was prevented by tying the arm to the trunk. During walk turning activity appeared in flexors and extensors which were inactive during straight walking The findings agree with the concept that the function of the arm swing is to counteract the rotation of the trunk and the pattern of innervation initiating it is considered to be part of a centrally determined locomotory pattern

We would like to thank chief mechanic Mr Vagn Andersen for his help in designing the telemetering circuit and Dr J C. Melchior who contributed the observations on arm movements during walking in children.

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Vasodilating Action of Substance P in the Human Forearm

By

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Received 27 June 1964

Abstract

Löfström B B Pernow and J Wahren *Vasodilating action of substance P in the human forearm* Acta physiol scand 1965 *63* 311—324 — The vasodilator activity of substance P in the human forearm has been studied using venous occlusion plethysmography The effects have been compared to those of bradykinin The compounds were given as single injections or infusions into the brachial artery or the antecubital vein I a administration caused a significant increase in skin and muscle blood flow in doses of more than 0 5 units of substance P per min The oxygen saturation of blood from both deep and superficial veins of the forearm increased These effects were not influenced by atropine antihistamines or guanethidine Stellate ganglion block increased the resting blood flow as well as the vasodilating action of both polypeptides. I v infusion of substance P and bradykinin also caused an increase in forearm blood flow coincident with tachycardia and flushing of the face and the neck During substance P infusion the increase in forearm blood flow and flushing appeared simultaneously while bradykinin caused an increase in the forearm blood flow only in doses which were almost intolerable for the subjects due to severe pulsations and flushing of the skin of the head and the neck The ratios between equieffective doses of substance P and bradykinin on the forearm blood flow were about 1 10 at i a infusion and about 1 200 at i v infusion

Already in the first publication on substance P (Euler and Gaddum 1931) it was suggested that the hypotensive effect obtained after i v administration of the substance was due to a peripheral vasodilatation Holton and Holton (1952) found an increased blood flow through the rabbit ear following i v injection of substance P During studies on the effect of substance P on the intestinal motility (Liljedahl Mattsson and Pernow 1958) and the central hemodynamics in man (Dunér and Pernow 1960) it was observed that i v infusion of the substance caused an intensive flushing in the face and neck obviously due to cutaneous vasodilatation

This paper deals with the effects of substance P following i a and i v administration on skin and muscle blood flow in the human forearm The effects of substance P

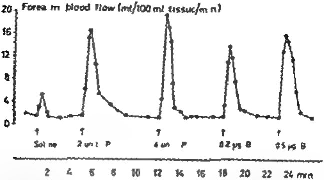


Fig. 1. Effect of single i.a. injections of substance P (P) and bradykinin (Br) of the forearm blood flow. The compounds are given in 0.1 ml saline. The effects of 0.1 ml saline alone is also shown.

are compared with those of bradykinin previously shown to be a very potent vasodilator in man (Fox *et al.* 1961, Burch and DePasquale 1962, Kontos *et al.* 1964) and very similar to substance P in its chemical structure (Boissonnas, Franz and Sturmer 1963).

Material and methods

The subjects were 13 healthy male volunteers, the age range being 21—41 years. A deep and a superficial vein and the brachial artery were catheterized percutaneously.

The forearm blood flow was measured with venous occlusion plethysmography according to Dohn (1956) as modified by Graf and Westersten (1959). The circulation of the hand was occluded by a cuff at the wrist. Using the same plethysmographic apparatus, Strandell and Wahren (1963) found a coefficient of variation of 12.5 per cent for a single flow curve registered on the calf.

The oxygen saturation of venous blood was determined spectrophotometrically using a slight modification of the methods described by Drabkin (1950) and Nahas (1951): the coefficient of variation, calculated from the analysis of two samples from forearm veins obtained within 5 min of each other, was 5.4 per cent.

For the stellate ganglion block the anterior or paratracheal approach was used (Moore 1954). The tip of the injection needle was placed just anterior to the transverse process of the C 7 vertebra. Twenty ml 0.5 per cent mepivacaine was injected. In all cases a Horner's syndrome appeared within 10 min. The completeness of the block was checked by testing the psychogalvanic reflex (Lewis 1955) and by the appearance of a significant increase in the resting blood flow of the forearm.

The following substances were used:

Substance P prepared from horse intestine by Hoffmann-La Roche AG according to the method described by Pernow (1953). The activity of the preparation was 200 Euler units per mg (Euler 1942).

Bradykinin, synthesized by Sandoz AG.

Atropine sulphate.

Promethazine chloride (Lergigan®, Recip).

Guanethidine bisulphate (Ismelin®, Ciba).

Acetylcholin hydrochloride (Hoffmann-La Roche AG).

Procedure

The investigation was performed with the subjects in the supine position. Teflon catheters were inserted percutaneously into the brachial artery and in some cases also into a deep and a superficial vein of the forearm. Infusions into the brachial artery or a cubital vein were per-

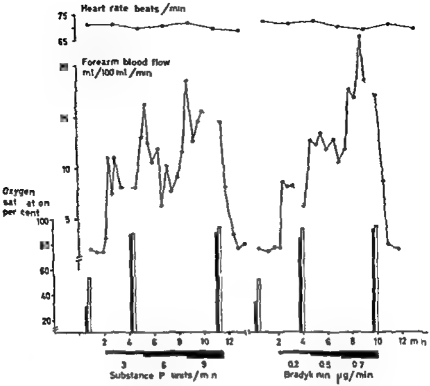


Fig 2 Effect of i.a infusion of substance P and bradykinin on heart rate forearm blood flow and oxygen saturation of deep (filled stems) and superficial (open stems) venous blood of the forearm

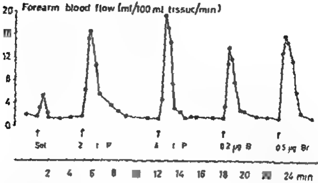
formed using a constant infusion pump with interchangeable gears and calibrated glass syringes. Blood samples for oxygen analysis were taken from a deep and a superficial vein of the forearm

Substance P and bradykinin were diluted in saline and given as infusion at a rate of 0.25—2.5 ml per min The volume of an i.a injection never exceeded 0.1 ml

Results

A Intraarterial administration

Blood flow Single injections of substance P in doses larger than 0.5 units gave a significant increase in the forearm blood flow The effect was very transient and the resting level was reached again approximately 1.5 min after the injection According to the finding by Vogler *et al* (1963) that the purest samples to date of substance P have an activity of about 120 000 units per mg 0.5 units corresponds to 4 ng Bradykinin in doses greater than 0 ng elicited an increase in the forearm blood flow similar in type and duration to that obtained with substance P Weight for weight comparisons of substance P and bradykinin at single injections showed that substance P was about 20—30 times more active as a vasodilator (Fig 1)



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Guanethidine bisulphate (Ismelin® Ciba)

Acetylcholin hydrochloride (Hoffmann La Roche AG)

Procedure

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flow (ml/min/100 ml) blood oxygen saturation of deep (DV) and superficial (SV) forearm solute weight calculated from an activity of 120 000 units per mg (Vogler et al 1963)

Bradykinin

Dose	Forearm blood flow ml/min/100 ml			Oxygen saturation per cent	
/g/min	1 min	2 min	3 min	DV	SV
—	1 6	2 5	2 2	46 9	[illegible]0 6
0 2	6 7	7 9	7 5		
0 7	13 8	8 8	9 3	87 [illegible]	9[illegible] 0
—	6 1	6 2	5 4	52 0	
0 0[illegible]	10 1	14 2	12 4		
0 07	22 4	23 6	24 0		
0 2	43 7	37 9	36 3	89 2	
—	2 7	2 6		68 0	
0 2	10 2	10 2			
0 7	10 6	8 8		92 2	
—	2 2	2 1	2 4		
0 2	10 7	16 2	15 8		
0 4	16 0	16 [illegible]	11 7		
—	1 6	1 9	2 1		
0 05	6 8	6 [illegible]	5 9		
0 1	16 8	12 5	9 9		
0 7	17 1	12 1	11 7		
—	2 4	2 1	2 6		
0 1	8 3	11 5	8 3		
0 7	18 6	14 4	12 1		
—	2 1	2 7	2 9	43 [illegible]	58 1
0 2	11 3	7 8	7 8		
0 7	31 1	26 0	23 5	88 9	88 3
—	2 4	2 7	2 4	31 0	80 1
0 03	2 4	2 7	3 0		
0 [illegible]	7 5	[illegible] 7	4 2	80 6	83 8
0 4	6 0	6 0	8 6		
0 7	11 9	16 1	15 5	91 6	93 9
—	1 7	1 9	1 9	34 0	52 1
0	8 6	8 3		84 1	9[illegible] 6
0 5	12 7	13 3	1[illegible] 9		
0 7	17 9	[illegible]3 2	18 8	91 7	[illegible]

Table I Cont.

Subject	Substance P — Dose units/min	μg/min	Forearm blood flow ml/min/100 ml 1 min	2 min	3 min	Oxygen saturation per cent DV	SV
JW	—	—	3.9	3.1	3.9	45.6	76.6
	5	0.04	17.4	9.8	8.6		
	25	0.2	15.9	18.8	19.4	81.2	94.7
DT	—	—	4.6	3.4	3.9	63.5	84.6
	1.5	0.01	10.1	8.0	8.4	86.4	91.1
	2.5	0.02	12.1	9.8	11.2	82.0	89.0
	5	0.04	12.4	14.3	11.9	78.0	28.0
SL	—	—	4.1	5.1	3.8		
	2.5	0.02	20.2	18.6	16.1		
	5	0.04	19.2	21.8	25.7		
	12.5	0.1	23.8	29.5	30.2		

A constant infusion of more than 0.5 units of substance P per min caused an increase [in] the blood flow which exceeded two times the standard deviation of the resting blood [fl]ow The individual sensitivity to substance P showed considerable variations Generally the maximal vasodilatation was obtained early during each infusion period where after it usually gradually decreased even during continued infusion If the infusion rate was then stepped up the blood flow increased further but usually not in proportion to the infusion rate (Fig 2 and Table I)

Bradykinin administered i a had a similar influence on the forearm blood flow as substance P 0.2 μg bradykinin gave about a threefold increase from the resting level and was roughly comparable to 2 units (16 ng) of substance P (Fig 2)

Oxygen saturation The oxygen saturation of blood samples from both deep and superficial forearm veins increased significantly during infusion of both substance P and bradykinin (Fig 2 and 3 Table I) In the deep vein the initial oxygen saturation values were 31—68 (mean 51 8) per cent these increased during infusion of 5—25 units of substance P to 78—90 (mean 85 1) per cent The corresponding values for the superficial vein were 52—87 (mean 71 7) per cent at rest and 89—95 (mean 92 2) per cent during substance P infusion. Similar results were obtained with bradykinin For both polypeptides the increase in blood oxygen saturation in deep and superficial veins was highly significant

Heart rate I a infusions of substance P and bradykinin which gave as much as a tenfold increase in forearm blood flow did not change the heart rate significantly (Fig 2 and 3)

Bradykinin					
Dose	Forearm blood flow ml/min/100 ml			Oxygen saturation per cent	
γg/min	1 min	2 min	3 min	DV	SV
—	3 8	2 8	3 4	42 1	77 3
0 2	7 8	6 1	7 7		
0 7	17 6	14 4	16 2	72 4	89 0
—	4 2	5 0	4 3	52 2	84 6
0 0?	11 5	11 0	8 7	78 0	92 5
0 1	10 4	9 1	7 8	77 6	94 6
0 2	13 4	9 3	11 3	76 5	93 1
—	6 1	5 8	6 0		
0 1	10 8	11 4	15 4		
0 2	11 8	15 4	17 5		
0 5	30 9	29 6	25 5		

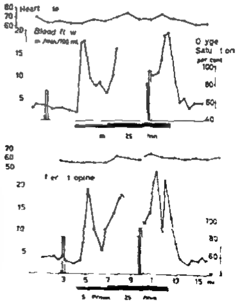


Fig 3 Influence of atropine on the effect of a infused substance P on the heart rate, forearm blood flow deep and superficial venous oxygen saturation Symbols as in Fig 2

TABLE II Effect of stellate block on the forearm blood flow response to intraarterial infusion period is given

Subject	Dose μg/min	Substance P: Blood flow ml/min/100 ml tissue, Before sympathetic block	Flow at infusion / basal flow	During sympathetic block	Flow at infusion / basal flow
KM	—	2 7		5 5	
	5	9 6	3 5	26 2	4 7
	25	13 6	5 0	39 6	7 2
GL	—	1 7		4 1	
	2 5	7 3	4 2	12 7	3 1
	5	8 8	5 1	19 6	4 7
	10	12 5	7 2	24 7	6 0
TH	—	2 0		3 8	
	1 5	9 2	4 6	13 9	3 6
	2 5	11 3	5 6	16 9	4 4
	5	17 2	8 6	23 2	6 1
	—	4 6		7 6	
	1 5	10 1	2 2	19 6	2 5
	2 5	12 2	2 6	15 8	4 0
	5	14 3	3 1	19 8	2 6
	—	1 8		6 7	
	1 5	6 1	3 3	19 2	2 8
	3 5	9 4	5 2	21 1	3 1
	7 5	13 6	7 5	230	3 4

Effect of atropine and antihistamine I a. injection of 0 5 mg atropine or 20 mg prome thazinechloride did not significantly influence the blood flow response to i a infused substance P or bradykinin (5 cases) (Fig 3)

Effect of guanethidine I a injection of 5 mg guanethidine did not cause a change in the resting blood flow or in the forearm blood flow to succeeding i a infusion of substance P (5 cases)

Effect of stellate block In 9 cases substance P and bradykinin were infused i. a before and during stellate block. *In 5 cases (Table II) the block led to a significant* increase in the forearm blood flow at rest and was accompanied by an extinguished sudomotor reaction following pain stimulation as judged by skin resistance measurements *In* these cases the increase in blood flow obtained with comparable doses of both substance P and bradykinin was invariably larger after the block than before (Fig 4 and *Table II*)

of substance P and bradykinin The maximal blood flow response recorded during each

Bradykinin				
	Blood flow ml/min/100 ml tissue			
	Before sympathetic block		During sympathetic block	
Dose /g/min		Flow at infusion / basal flow		Flow at infusion / basal flow
—	2 5		5 8	
0 2	7 9	3 1	21 0	3 6
0 7	13 7	5 4	25 1	4 3
—	2 5		5 5	
0 2	15 4	6 1	26 5	4 8
0 4	16 5	6 6	26 8	4 8
—	2 0		9 2	
0 05	6 3	3 1	12 4	1 3
0 1	16 7	8 3	30 0	3 2
0 7	17 1	8 6	35 2	3 8
—	5 0		6 3	
0 02	11 5	2 0	15 6	2 4
0 1	10 4	2 0	14 3	2 2
0 ?	13 4	2 6	16 0	2 5

In the other 4 cases in which the stellate block was incomplete as indicated by a negative sudomotor test and no increase in forearm blood flow at rest both substance P (Fig 5) and bradykinin had almost identical effects before and after the block

B Intravenous administration

In doses larger than 10 units per min substance P elicited a significant increase in the forearm blood flow 50 units per min gave an eight to tenfold increase in blood flow No further increase was obtained however when the infusion rate was raised still further This phenomenon is illustrated in Fig 6 which shows that maximal blood flow response was reached about 2 min after starting an infusion of 50 units per min During continued infusion the blood flow then invariably decreased slowly though it rose again when the infusion rate was doubled In some cases the flow then exceeded that with the

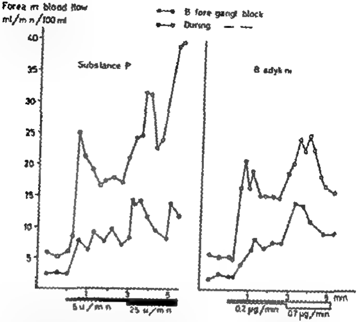


Fig 4 Effect of complete stellate block on the forearm blood flow response to i a infused substance P (left) and bradykinin (right) in subject K M

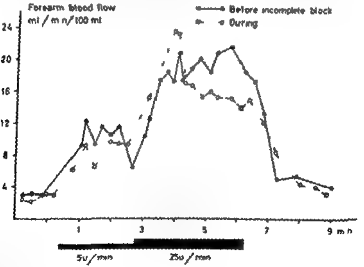


Fig 5 Effect of incomplete stellate block on the forearm blood flow response to i a infusions of substance P

smaller dose such as the one illustrated in Fig 6 and 7 it never even reached the previous maximum

The heart rate increased when substance P was given i v in doses larger than 25 units per min

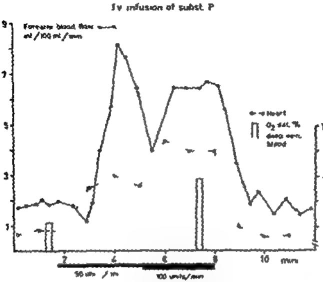


Fig 6 Effect of i v infusion of substance P on the forearm blood flow heart rate and oxygen saturation of deep venous blood

The i v infusions of substance P of 50 units per min or more were experienced by the subjects as a feeling of warmth in the head neck and arms where a bright red flush was also clearly noticeable The subjective symptoms disappeared almost immediately when the infusion was stopped

Bradykinin did like substance P cause an increase in the forearm blood flow when infused i v This effect was, however obtained only with very high doses (~ 100 μg per min) (Fig 7) causing intense and almost intolerable subjective symptoms of pulsations and a feeling of warmth in the head

Discussion

It is clear from these results that substance P as well as bradykinin is an extremely powerful vasodilator in skin and muscle of man Comparisons with other substances having a similar effect show it to be the most potent in this respect A direct weight for weight comparison is complicated by the varied reports on the biological activity of substance P as purified by different research teams Thus Franz Boissonnas and Sturmer (1961) gave an activity of 30 000 Euler units per mg for their preparation which they regard as chemically pure whereas Vogler *et al* (1963 report an activity of 170 000 units per mg The discrepancy may be due to differences in the standard substances used From these figures, it would seem that substance P is 5—20 times more effective than bradykinin on i a infusion and considerably more effective than acetylcholine as a vasodilator in the forearm region

The increase in forearm blood flow was accompanied by a significant rise in the oxygen saturation of blood from deep as well as superficial forearm veins This finding

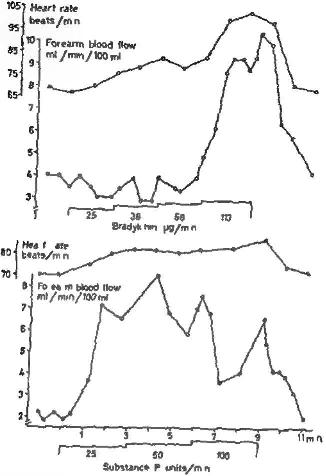


Fig 7 Effects of i v infusions of bradykinin (upper part) and substance P (lower part) on heart rate and forearm blood flow

seems to indicate an increase in both muscle and skin blood flow as substance P is considered not to affect the oxygen consumption of the tissue

It seems reasonable to suppose that the vasodilatation arises from a direct dilatating effect of substance P on the smooth muscle of the blood vessels The negative results with antihistamines and atropine should rule out transmission of the effect via histamine or acetylcholine

After sympathetic block when release of vasoconstrictor tone had caused an increase in resting blood flow both substance P and bradykinin had a more marked effect In the case of bradykinin this finding agrees with the observations of Rocha e Silva Corrado and Ramos (1960) that substances with a sympathicolytic action such as dibenzyline delay the recovery from hypotension provoked by small doses of bradykinin

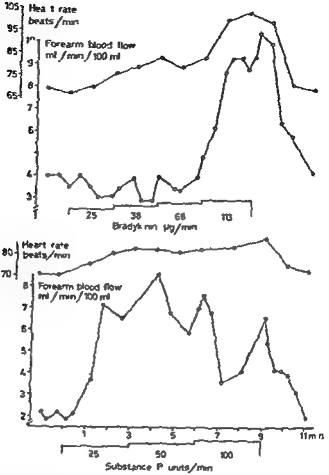
It is difficult to assess the more marked vasodilatating effect following sympathetic block however as such blocking in itself changes the initial tonus of the resistance

vessels It is likely that the effect of vasodilating drugs is to a large extent dependent on the initial tone of the vessel If however, a constant relaxing effect on the vessel circumference is assumed for a given dose of a vasodilator regardless of the initial tone Poiseuille s law indicates a greater absolute increase in flow following administration at a low initial tone than at a high one The effect of the given polypeptides should accordingly be evaluated as the ratio between the initial blood flow and the flow as measured during the infusion of a particular dose In the 5 cases examined the absolute increase in blood flow during infusion of substance P and bradykinin was always greater after sympathetic block Only in one case (K. M Fig 4) however was the ratio between the flow during infusion and the initial flow greater after the block in this case the results might suggest that substance P actually had a greater effect after blocking In the other cases the greater increase in flow after blocking is largely ascribable to the change in the initial tonus and must not necessarily mean a potentiation of the vasodilating action following sympathetic block

While the normal forearm blood flow is fairly stable it showed great variations during constant infusion of substance P and bradykinin (see Fig 2 4 and 5) This might be due to an uneven distribution of the infused polypeptides in the arterial tree of the forearm However since the same pattern of flow response occurs at i v infusion (Fig 7) this explanation seems unlikely During prolonged infusion of substance P or bradykinin the maximal effect always appeared during the first minute after which the flow gradually decreased A further increase was generally obtained when the infusion rate was increased though this rise was not directly proportional to the amount infused A true dose response curve was therefore never obtained with these compounds A few cases showed so marked a decline in the "sensitivity" to further doses of substance that the infusion rate could be multiplied several times following the administration of a small dose for a few minutes without any further recordable increase in the blood flow This was particularly evident during i v infusion Similar findings have been reported for bradykinin (Kontos *et al* 1964) and also for other vasoactive substances such as adrenaline (Whelan 1956) The cause of this change in the response to a substance during prolonged administration is not yet known It might be supposed that the vasodilatation induced elicits a sympathetic vasoconstriction. Such an explanation seems improbable however in view of the finding that the same phenomenon occurs when substance P or bradykinin is infused after sympathetic block (Fig 4) It seems more likely that the phenomenon represents a tachyphylaxis of the same type as that previously demonstrated for the effect of substance P on the intestine (Gaddum 1953)

Obviously substance P is much more active than bradykinin as a vasodilator of the forearm on i v administration This is in accordance with the findings by Fox (1961) who also found that bradykinin infused i v caused a vasodilatation of the face and neck at a dose level which was too low to give any effect on the forearm. This was not the case with substance P which caused a simultaneous increase in forearm blood flow and flushing of the face While the mean ratio of equi-effective doses of substance P and bradykinin as vasodilators of the forearm was about 1 10 at i a infusion the corresponding ratio at i v infusion was about 1 200

This work was supported by grants from the Swedish Medical Research Council and the Swedish Society for Medical Research which are gratefully acknowledged



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From the Institute for Medical Biochemistry University of Oslo Norway

Effect of Insulin on the Isolated Rat Diaphragm in the Presence and in the Absence of Puromycin and Actinomycin D[1]

By

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Abstract

Sovik O *Effect of insulin on the isolated rat diaphragm in the presence and in the absence of puromycin and actinomycin D* Acta physiol scand 1965 *63* 325—335 — To determine if insulin-induced sugar uptake and glycogen synthesis in isolated muscle tissue are dependent upon RNA or protein synthesis the effect of puromycin and actinomycin D was studied on these parameters At a concentration of puromycin which inhibited by 97 the incorporation of C-protein hydrolysate into proteins an inhibition of the insulin stimulated incorporation of [14]C-glucose into glycogen and of the net glycogen synthesis was observed This puromycin effect was demonstrated in the cut as well as in the intact diaphragm preparation, and at low as well as high glucose concentrations in the incubation medium The insulin induced glucose uptake and D-xylose transport was not altered by puromycin Actinomycin D at a concentration which almost blocked the incorporation of C-orotic acid into RNA had no inhibitory effect on glucose uptake or glycogen synthesis. At a higher concentration of actinomycin D where incorporation of labelled amino acids into proteins was strongly inhibited insulin stimulation of glycogen synthesis was almost abolished The results favour the view that synthesis of messenger RNA is not involved in the insulin effect on muscle The relationship between protein synthesis and insulin induced glycogen synthesis is discussed

Insulin when added to the isolated rat diaphragm, stimulates glucose uptake and glycogen synthesis (Gemmill and Hamman 1941) These effects of insulin have been related to an enhanced rate of membrane transport of glucose (Levine *et al* 1950 Park and Johnson 1955) however the precise mechanism of this "membrane effect remains unknown

Experiments have favoured the hypothesis that the insulin effect on glycogen synthesis is not only a consequence of increased entry of glucose into cells but also depends upon a pathway to glycogen specifically facilitated by insulin (Beloff Chain *et al* 1956 Norman *et al* 1959 Larner *et al* 1960) It has been suggested that this "direc

[1] A preliminary report of this work has been published in *Nature* 1964 *0* 396—397

tive effect on glycogen synthesis depends upon an increase in the activity of glycogen synthetase following exposure of the muscle to insulin (Villar Palasi and Larner 1961).

It has been demonstrated that insulin produces an effect on protein biosynthesis in muscle (Krahl 1952, Sinex *et al.* 1952), independent of the insulin effect on membrane transport of sugars and amino acids (Wool and Krahl 1959), and recently Wool (1963) has observed an effect of insulin on nucleic acid synthesis in isolated rat diaphragm.

In the present work it has been investigated whether insulin action on carbohydrate metabolism is related to the stimulation of protein and RNA biosynthesis exerted by this hormone. Therefore, the effect of insulin on glucose uptake, xylose distribution and glycogen synthesis was studied under conditions where protein and RNA biosynthesis was blocked by puromycin and actinomycin D, respectively. Puromycin is known as a specific inhibitor for protein biosynthesis in mammalian cells (Yarmolinsky and de la Haba 1959). In presence of actinomycin D a selective and complete inhibition of cellular RNA synthesis has been observed (Reich *et al.* 1962).

Materials and methods

ANIMALS

Male and female rats (100—160 g) from the strain at the institute were kept on standard diet. Before the diaphragms were removed, the animals were fasted 18—24 hours, then killed by a blow on the head and decapitated.

MATERIALS

Puromycin-di hydrochloride (purchased from the Nutrition Biochem. Corp.) and actinomycin D (a gift from Dr. Mushett, Merck Sharp & Dohme) were weighed and dissolved in buffer to the appropriate concentrations before each experiment. Insulin crystalline (23.4 units/mg), D(+)xylose and sucrose were purchased from The British Drug House. Every ten days a stock solution was made by dissolving insulin in 0.6 % acetic acid to a concentration of 50 units/ml. [illegible]dase and glucose oxidase were purchased from Sigma, p-bromanilin from Dr. Theodor Schuchardt, Munich, thiourea and resorcinol from Merck, D-glucose from Difco Lab., Detroit, U-^{14}C-glucose, ^{14}C-protein hydrolysate and ^{14}C-orotic acid from The Radiochemical Centre, Amersham, and ^{14}C-DL-Phenylalanine from Biochem. Research Corp., Los Angeles. The specific activity of ^{14}C-glucose was 19.6 µC/mg, of ^{14}C-protein hydrolysate [illegible]00 µC/mg, of ^{14}C-orotic acid 60.7 µC/mg and of ^{14}C-DL-phenylalanine 12 mC/mM.

INCUBATION TECHNIQUES

Four—eight rats were used for each experiment.

A. Experiments with cut diaphragm

Cut diaphragms were prepared, weighed and subjected to preincubation, one hemidiaphragm in 1 ml buffer with inhibitor (puromycin or actinomycin D), the other hemidiaphragm was kept in buffer as a control. Krebs-Ringer phosphate buffer, pH 7.2—7.4, gassed with O_2 was used. Preincubation was performed for 15—30 min at room temperature (22° C). Thereupon sugars, *insulin and ^{14}C-labelled compounds were added to the appropriate concentrations* and the vessels stoppered, gassed with O_2 for 5 min and incubated for 1—2 hrs at 37° C. Incubation was performed in a Dubnoff shaker with 80—100 cycles/min. After incubation the diaphragms were blotted *on filter paper and* subjected to the analytical procedures mentioned later.

B. Experiments with "intact" diaphragm

"Intact" diaphragms were prepared according to Kipnis and Cori (1957) and divided along the midline (Kono and Colowick 1961). The hemidiaphragms were each preincubated and incubated in 6 ml Krebs-Ringer phosphate buffer in the same manner as the cut preparation. After incubation the "intact" preparations were blotted on filter paper, the diaphragm muscle cut out, trimmed, weighed and subjected to the analytical procedures.

TABLE I Effect of puromycin on labelling of protein in the isolated rat diaphragm in the presence and in the absence of insulin Paired intact hemidiaphragms were preincubated for 30 min and incubated for 2 hrs with or without puromycin (270 μg/ml) Hemidiaphragms from every second animal were incubated with or without insulin (0 1 unit/ml) The incubation medium (6 ml) contained glucose (200 mg/100 ml) and 0 33 μC of C-labelled precursor per ml Proteins were isolated and counted as described under analytical procedures

Additions to incubation medium	Number of experiments	Rate of incorporation of C-labelled amino acids into proteins	
		Precursor C DL phenylalanine	Precursor C protein hydrolysate
		Counts/min/100 mg wet tissue[1]	Counts/min/100 mg wet tissue
No addition	4	1 376 $\pm$ 365	1 376 $\pm$ 50
Puromycin	4	159 $\pm$ 65	68 $\pm$ 5
Insulin	4	2 848 $\pm$ 875	2 223 $\pm$ 585
Insulin + puromycin	4	241 $\pm$ 96	84 $\pm$ 11

$\pm$ Standard error of the mean

ANALYTICAL PROCEDURES

Glucose was determined by the glucose oxidase method (Huggett and Nixon 1957) Glycogen was isolated according to Walaas and Walaas (1950) with the modification that the tissue was boiled in 30 NaOH instead of 30 KOH Glycogen determination was performed by the anthrone method of Carroll *et al* (1956) Net glycogen synthesis was calculated from a mean fasting glycogen level This was obtained by cutting small pieces from the diaphragms sampling the pieces and estimating the glycogen content of the pooled sample Samples of C labelled glycogen dissolved in water were applied on plates and counted in a windowless methane flow counter (Frieseke and Hoepfner) Proteins were isolated according to Manchester and Young (1958) applied on plates and counted in the same manner as described for glycogen Nucleic acids were isolated as described by Wool (1963) During an incubation time of 2 hours C orotic acid was almost exclusively incorporated into RNA Therefore a specific determination of RNA and DNA was not performed for the bulk of the experiments D-xylose was determined by the method of Roe and Rice (1948) and sucrose according to Roe *et al* (1949 The tissue distribution of a sugar was obtained by the ratio

$$\frac{\text{sugar in wet tissue (mg/g)}}{\text{sugar in medium (mg/ml)}} \quad 100$$

The intracellular distribution of xylose [illegible] the difference between the total xylose distribution and the sucrose distribution Sucrose distribution is limited to the extracellular phase while D xylose penetrates cell membranes

Results

Experiments with puromycin

The effect of puromycin on protein biosynthesis was tested In the presence of puromycin (270 μg ml) there was a strong inhibition of incorporation of C-DL phenylalanine and C-protein hydrolysate into proteins of the diaphragms Table I) The

tive effect on glycogen synthesis depends upon an increase in the activity of glycogen synthetase following exposure of the muscle to insulin (Villar Palasi and Larner 1961)

It has been demonstrated that insulin produces an effect on protein biosynthesis in muscle (Krahl 1952 Sinex *et al* 1952) independent of the insulin effect on membrane transport of sugars and amino acids (Wool and Krahl 1959) and recently Wool (1963) has observed an effect of insulin on nucleic acid synthesis in isolated rat diaphragm

In the present work it has been investigated whether insulin action on carbohydrate metabolism is related to the stimulation of protein and RNA biosynthesis exerted by this hormone Therefore the effect of insulin on glucose uptake xylose distribution and glycogen synthesis was studied under conditions where protein and RNA biosynthesis was blocked by puromycin and actinomycin D respectively Puromycin is known as a specific inhibitor for protein biosynthesis in mammalian cells (Yarmolinsky and de la Haba 1959) In presence of actinomycin D a selective and complete inhibition of cellular RNA synthesis has been observed (Reich *et al* 1962)

Materials and methods

ANIMALS

Male and female rats 100—160 g from the strain at the institute were kept on standard diet Before the diaphragms were removed the animals were fasted 18—24 hours then killed by a blow on the head and decapitated

MATERIALS

Puromycin-dihydrochloride purchased from the Nutrition Biochem Corp and actinomycin D a gift from Dr Muchett Merck Sharp & Dohme were weighed and dissolved in buffer to the appropriate concentrations before each experiment Insulin crystalline (23 4 units mg) D(+)xylose and sucrose were purchased from The British Drug House Every ten days a stock solution was made by dissolving insulin in 0 6 acetic acid to a concentration of 50 units ml Peroxidase and glucose oxidase were purchased from Sigma p bromoanilin from Dr Theodor Schuchardt Munich thiourea and resorcinol from Merck D glucose from Difco Lab Detroit U ^{14}C-glucose ^{14}C-protein hydrolysate and ^{14}C-orotic acid from The Radiochemical Centre Amersham and ^{14}C-DL-Phenylalanine from Biochem Research Corp Los Angeles The specific activity of ^{14}C glucose was 19 6 μC/mg of ^{14}C proteinhydrolysate 700 μC/mg of ^{14}C orotic acid 66 7 μC/mg and of ^{14}C DL phenylalanine 1? mC/mM

INCUBATION TECHNIQUES

Four—eight rats were used for each experiment

A Experiments with cut diaphragm

Cut diaphragms were prepared weighed and subjected to preincubation one hemidiaphragm in 1 ml buffer with inhibitor (puromycin or actinomycin D) the other hemidiaphragm was kept in buffer as a control Krebs Ringer phosphate buffer pH 7 ?—7 4 gassed with O was used Preincubation was performed for 15—30 min at room temperature (2? C) Thereupon sugars insulin and ^{14}C-labelled compounds were added to the appropriate concentrations and the vessels stoppered gassed with O for 5 min and incubated for 1—? hrs at 37 C Incubation was performed in a Dubnoff shaker with 80—100 cycles min After incubation the diaphragms were blotted on filter paper and subjected to the analytical procedures mentioned later

B Experiments with intact diaphragm

Intact diaphragms were prepared according to Kipnis and Cori (19?7) and divided along the midline (Kono and Colowick 1961) The hemidiaphragms were each preincubated and incubated in 6 ml Krebs-Ringer phosphate buffer in the same manner as the cut preparation After incubation the intact preparations were blotted on filter paper the diaphragm muscle cut out trimmed weighed and subjected to the analytical procedures

I *Effect of insul n on xylose distribution in isolated intact rat diaphragm in the* presence and in the absence of puromycin Paired intact' hemidiaphragms were preincubated for 30 min and incubated for 30 min with or without puromycin (270 µg/ml) Hemidiaphragms from every second animal were incuba ed w th or without insulin (0 1 unit/ml) The incubation medium (6 ml) contained D-xylose (450 mg/100 ml) and sucrose (685 mg/100 ml) Xylose and sucrose were assayed and xylose distribution calculated as described under analytical procedures

to incubation	Number of experiments	Intracellular xylose distribution
n	6	20 37 ± 1 69
	6	17 93 ± 1 78
	6	32 83 ± 1 30
uromycin	6	28 17 ± 3 02

ard error of the mean
I

ffect of insulin on net glycogen synthes s and incorporation of C glucose into lycog n n isolated ntact rat d aphragm in the presence and in the absence f puromycin Paired intact hemid aphragms were preincubated for 15—30 min nd incubated for 1 hr w th or w thout puromycin (270 µg/ml) Hemidiaphragms om every second an mal were incub ted with or without insulin (0 1 unit/ml) he incubation medium (6 ml) contained glucose (200 mg/100 ml) and C-glucose 4 µC/ml) Glycogen was isolated as described under analytical procedures ne aliquot was used for ca boh drate estimation by the anth one procedure an ner was counted on plates

Number of experi ments	Net glycog n synthesis	Rate of incorporation of C glucose into glycogen	Specific activity of glycogen
	mg g wet tissue	Counts/min/mg wet tissue	Counts/min/µg
6	1 21 ± 0 22	99 ± 11	37 8 ± 5 1
[illegible]	1 19 ± 0 30	170 ± [illegible]	46 7 ± 8 3
9	3 73 ± 0 3[illegible]	503 ± 3[illegible]	96 4 ± 4 7
9	3 03 ± 0 37	431 ± 28	9[illegible] 4 ± 4 1

r of the mean

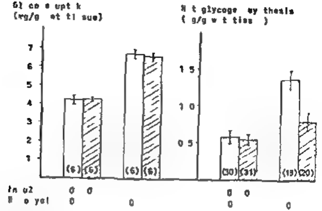

Fig 1 *Effect of insulin on glucose uptake and net glycogen synthesis in isolated "cut" rat diaphragm in the presence and in the absence of puromycin*
Paired "cut" hemidiaphragms were preincubated for 15 min and incubated for 1 hour with or without puromycin (270 μg/ml) The incubation medium (1 ml) contained glucose (200 mg/100 ml) Glucose uptake and net glycogen synthesis were estimated as described under "analytical procedures" No of experiments indicated in parentheses and standard error of the mean indicated on the top of each column

inhibition of incorporation of C-proteinhydrolysate was about 97 % in the absence as in the presence of insulin Insulin enhanced the incorporation of amino acids into proteins by about 100 %

Fig 1 shows the influence of insulin and puromycin on glucose uptake With insulin the glucose uptake was stimulated by approximately 75 % There was no effect of puromycin on glucose uptake neither in the absence nor in the presence of insulin

In Table II are shown experiments with D xylose a non metabolized sugar The intracellular distribution of D xylose was unchanged after the addition of puromycin In presence of insulin the intracellular xylose distribution was increased This stimulation by insulin was not significantly altered by puromycin

The influence of insulin and puromycin on glycogen synthesis was tested (Fig 1) *In experiments with "cut" diaphragm puromycin without added insulin had* no effect on glycogen synthesis Addition of insulin more than doubled the glycogen synthesis This insulin induced glycogen synthesis however was reduced by puromycin While the insulin stimulation of glycogen synthesis was 134 % without puromycin in the incubation medium insulin stimulation was reduced to 44 % in the presence of puromycin

The influence of insulin and puromycin on the "intact" diaphragm preparation was investigated (Table III) In the "intact" preparation the insulin response was more marked than in the "cut" preparation Insulin stimulated the net glycogen synthesis by about 200 % and the incorporation of C-glucose into glycogen by about 400 % There was a marked increase in the specific activity of glycogen after addition of insulin Puromycin did not affect the net glycogen synthesis or the incorporation of ^{14}C-glucose into glycogen in the absence of insulin However the insulin effect on glycogen synthesis was somewhat reduced by puromycin *In presence of puromycin* the insulin effect on net glycogen synthesis was reduced from about 200 % to about

TABLE II Effect of insulin on xylose distribution in isolated "intact" rat diaphragm in the presence and in the absence of puromycin. Paired "intact" hemidiaphragms were preincubated for 30 min and incubated for 30 min with or without puromycin (270 µg/ml). Hemidiaphragms from every second animal were incubated with or without insulin (0.1 unit/ml). The incubation medium (6 ml) contained D-xylose (450 mg/100 ml) and sucrose (685 mg/100 ml). Xylose and sucrose were assayed and xylose distribution calculated as described under "analytical procedures".

Additions to incubation medium	Number of experiments	Intracellular xylose distribution
No addition	6	20.37 ± 1.69
Puromycin	6	17.93 ± 1.78
Insulin	6	3? .83 ± 1.30
Insulin + puromycin	6	28.17 ± 3.02

± Standard error of the mean
p < 0.001
p < 0.02

TABLE III Effect of insulin on net glycogen synthesis and incorporation of C-glucose into glycogen in isolated "intact" rat diaphragm in the presence and in the absence of puromycin. Paired "intact" hemidiaphragms were preincubated for 15—30 min and incubated for 1 hr with or without puromycin (270 µg/ml). Hemidiaphragms from every second animal were incubated with or without insulin (0.1 unit/ml). The incubation medium (6 ml) contained glucose (200 mg/100 ml) and C-glucose (0.4 µC/ml). Glycogen was isolated as described under "analytical procedures". One aliquot was used for carbohydrate estimation by the anthrone procedure, another was counted on plates.

Additions to incubation medium	Number of experiments	Net glycogen synthesis	Rate of incorporation of C glucose into glycogen	Specific activity of glycogen
		mg/g wet tissue	Counts/min/mg wet tissue	Counts/min/µg
No addition	6	1.71 ± 0.[illegible]	99 ± 11	3[illegible].8 ± [illegible].1
Puromycin	6	1.19 ± 0.30	120 ± 2	46.7 ± 8.3
Insulin	9	3.[illegible]3 ± 0.3	[illegible]03 ± 3[illegible]	96.4 ± 4.7
Insulin + puromycin	9	3.03 ± 0.3	431 ± [illegible]8	9[illegible].4 ± 4.1

± Standard error of the mean
and p 0.0

TABLE IV Effect of insulin on glucose uptake net glycogen synthesis and incorporation of ^{14}C-glucose into glycogen in cut rat diaphragm in the presence and in the absence of puromycin Glucose concentration in incubation medium 600 mg/100 ml Paired cut hemidiaphragms were preincubated for 30 min and incubated for 1 hr with or without puromycin (270 μg/ml) Hemidiaphragms from every second animal were incubated with or without insulin (0 1 unit/ml) The incubation medium (1 ml) contained glucose (600 mg/100 ml) and C-glucose (0 5 μC/ml) Glucose uptake net glycogen synthesis and labelling of glycogen were determined as described under analytical procedures Number of experiments in parantheses

Additions to incubation medium	Glucose uptake	Net glycogen synthesis	Rate of incorporation of ^{14}C-glucose into glycogen
	mg g wet tissue	mg g wet tissue	Counts min mg wet tissue
No addition	7 13±0 27 (11)	2 56±0 ?3 (12)	2?7±1? (12)
Puromycin	6 30 ±0 45 (12)	2 50±0 15 (12)	210±16 (1?)
Insulin	11 52±1 3 (14	?11±0 38 (11)	412±?3 (1?
Insulin + puromycin	10 80±1 01 (14	*4 41±0 35 (11)	332±?2 (1?)

± Standard error of the mean
■ < 0 05
p < 0 05

TABLE V Effect of actinomycin D on labelling of RNA and proteins in the isolated rat diaphragm Paired cut hemidiaphragms were preincubated for 30 min and incubated for ? hrs with or without actinomycin D 10 μg ml The incubation medium contained glucose (200 mg 100 ml and C-orotic acid 0 2 μC ml or ^{14}C protein hydrolysate (0 4 μC ml RNA and proteins which were determined in different experiments, were isolated as described under analytical procedures Number of experiments in parentheses

Additions to incubation medium	Rate of incorporation of ^{14}C orotic acid into RNA	Rate of incorporation of ^{14}C protein hydrolysate into protein
	Counts min 100 mg wet tissue	Counts min 100 mg wet tissue
No addition	230 – ?8 4	1 183 ± 1?1 11
Actinomycin D	?1 8 4	838 11

* ± Standard error of the mean
* p < 0 01

TABLE VI Effect of insulin on glucose uptake net glycogen synthesis and incorporation of C-glucose into glycogen in cut rat diaphragm in the presence and in the absence of actinomycin D Paired cut hemidiaphragms were preincubated for 30 min and incubated for 1 hour with or without actinomycin D Hemidiaphragms from every second animal were incubated with or without insulin (0 1 unit/ml) The incubation medium contained glucose (200 mg/100 ml) and C glucose (0 5 μC ml) Glucose uptake net glycogen synthesis and labelling of glycogen were determined as described under analytical procedures

Additions to incubation medium	Number of experiments	Glucose uptake	Net glycogen synthesis	Rate of incorporation of C-glucose into glycogen
		mg g wet tissue	mg g wet tissue	counts min mg wet tissue
A Actinomycin D 10 μg ml				
No addition	6	3 78 ± 0 33	1 42 ± 0 50	329 ± 32
Actinomycin D	6	4 02 ± 0 19	1 71 ± 0 56	356 ± 46
Insulin	6	[illegible]17 ± 0 53	3 [illegible]0 ± 0 47	663 ± 5
Insulin + actinomycin D	6	6 61 ± 0 05	3 63 ± 0 69	66[illegible] ± 73
B Actinomycin D 100 μg ml				
No addition	6	*2 [illegible]0 ± 0 18	2 06 ± 0 [illegible]5	380 ± 24
Actinomycin D	6	3 84 ± 0 10	[illegible] 4[illegible] ± 0 4	499 ± 46
Insulin	6	6 96 ± 0 48	4 58 ± 0 35	790 ± 31
Insulin + actinomycin D	6	5 98 ± 0 44	[illegible] 6[illegible] ∓ 0 35	581 ± 60

± Standard error of the mean
p < 0 001
p < 0 01
p < 0 05

50 (p < 0 05) while the insulin effect on the rate of incorporation of C-glucose was reduced from about 400 to about 260 (p < 0 05) No effect of puromycin on the specific activity of glycogen was observed

It was of interest to study the effect of insulin and puromycin on glycogen synthesis at a higher glucose concentration in the incubation medium (Table IV Glucose uptake as well as net glycogen synthesis could be stimulated by increasing the glucose concentration in the incubation medium from 200 mg 100 ml to 600 mg 100 ml The stimulation of net glycogen synthesis produced in this way was not reduced by puromycin The effect of insulin on glucose uptake and net glycogen synthesis was of a similar order at the two glucose concentrations Fig 1 and Table IV) At the higher glucose concentration insulin raised the rate of incorporation of C-glucose into glycogen

by about 80 %. In presence of puromycin this insulin effect was somewhat reduced ($p < 0.05$). Puromycin did not significantly alter the insulin effect on net glycogen synthesis.

Experiments with actinomycin D

A. Actinomycin D 10 µg/ml

At this concentration a complete block was observed in the incorporation of ^{14}C-orotic acid into RNA of the diaphragm (Table V). At the same time a significant inhibition of the rate of incorporation of ^{14}C-protein hydrolysate into muscle protein was observed. Actinomycin D had no effect on glucose uptake, net glycogen synthesis or rate of incorporation of ^{14}C-glucose into glycogen, neither in the absence nor in the presence of insulin (Table VI).

B. Actinomycin D 100 µg/ml

As in the case above actinomycin D had no effect on net glycogen synthesis or rate of incorporation of ^{14}C-glucose without added insulin (Table VI). With insulin in the incubation medium an increase of net glycogen synthesis of 120 % was observed. In the presence of actinomycin D this insulin effect was almost abolished (less than 10 %). Insulin stimulated the rate of incorporation of ^{14}C-glucose into glycogen by about 110 %. In presence of actinomycin D this insulin effect was reduced to less than 20 %.

At the higher concentration of actinomycin D an effect on glucose uptake was observed (Table VI). Without addition of insulin there was a significant increase in glucose uptake of the diaphragm.

With actinomycin D 100 µg/ml a more marked effect on protein biosynthesis has been observed. The 30 % inhibition observed with actinomycin D 10 µg/ml (Table V) was raised to about 50 % at the higher concentration of actinomycin D.

Discussion

The effect of insulin on membrane transport of sugars and amino acids does not seem to be dependent on protein synthesis. Eboué-Bonis et al. (1963) in their studies on isolated rat diaphragm found no effect of puromycin on glucose uptake, neither in the absence nor in the presence of insulin. Fritz and Knobil (1963) could produce an in vitro stimulation by insulin of α-aminoisobutyrate transport in the absence of protein synthesis. Carlin and Hechter (1964) demonstrated the lack of effect of puromycin upon insulin induced D-xylose transfer and α-aminoisobutyrate transport in the intact diaphragm preparation. The observations concerning glucose uptake and D-xylose transfer are in accordance with the results presented here.

In the present work it has been observed that at a concentration of puromycin which almost blocks protein synthesis, but does not influence the effect of insulin on glucose uptake, the insulin stimulation of glycogen synthesis is partly inhibited. In contrast to this observation Carlin and Hechter (1964) conclude from their studies on the intact diaphragm preparation that puromycin in concentrations which almost abolish peptide bond synthesis do not influence insulin action upon glycogen synthesis. However, it is apparent from the results of these authors that while puromycin is without effect on the insulin induced net glycogen synthesis, the same inhibitor tends to reduce the insulin-stimulated incorporation of ^{14}C-glucose into glycogen. In the present work a difference between the cut and the intact diaphragm preparation has been

observed. The effect of puromycin on the insulin induced net glycogen synthesis in the intact diaphragm preparation was of marginal statistical significance ($p < 0.05$) while in the cut preparation there was a more marked effect of puromycin The reason for this is not clear It might be due to a higher intracellular concentration of puromycin in the cut preparation In this connection it is of interest to note that Carlin and Hechter (1964) used a puromycin concentration of 0.11 mM which was sufficient to produce more than 90 % inhibition of protein synthesis In the present work a concentration of 270 μg/ml (= 0.5 mM) was used This inhibited protein synthesis by 97 % Eboué Bonis *et al* (1963) in their work on cut rat diaphragm used an even higher concentration of puromycin (500 μg/ml)

As mentioned previously in this paper observations by Beloff Chain *et al* (1956) Norman *et al* (1959) and Larner *et al* (1960) have favoured the view that insulin has a directive effect on glycogen synthesis independent of its effect on membrane transport of glucose. In connection with a puromycin inhibition of insulin induced glycogen synthesis it should be mentioned that insulin influences the activity of enzymes concerned with glycogen synthesis Villar Palasi and Larner (1961) found an increase in the activity of glycogen synthetase (UDP glucose α 1 4 glucan-α-4-glucosyl transferase) after exposure of isolated rat diaphragm to insulin When the enzyme was fully stimulated by addition of glucose-6-phosphate there was no further effect of insulin. It was therefore difficult to attribute the effect of insulin to a *de novo* synthesis of enzyme Later Rosell Perez and Larner (1962) demonstrated the existence of two forms of this enzyme and postulated that insulin acts to regulate their interconversion

Observations on liver however suggest an insulin induced *de novo* synthesis of enzymes Steiner *et al* (1961) found an increase in the activity of glycogen synthetase in liver homogenates from alloxan diabetic rats within two hours after insulin injection This insulin effect was present both with and without added glucose-6-phosphate In diabetic rats Salas *et al* (1963) could restore glucokinase activity by insulin administration. Changes in some metabolite affecting the activity of glucokinase could not be demonstrated and it was suggested that glucokinase synthesis was induced directly or indirectly by insulin Similar results were obtained by Sharma *et al* (1963) who postulated that hepatic glucokinase requires insulin for its adaptive synthesis

According to Reich *et al* (1962) actinomycin D at appropriate concentrations selectively suppresses RNA synthesis in animal cells Concentrations of actinomycin D which diminish RNA synthesis by 99 permit substantial rates of protein synthesis for prolonged periods At higher concentrations of actinomycin D however biosynthesis of DNA and protein is inhibited

The effect of actinomycin D on isolated rat diaphragm has been investigated by Eboué Bonis *et al* (1963) At a concentration of actinomycin D (10 μg/ml) which blocked the biosynthesis of RNA there was no effect of insulin on the incorporation of C-labelled amino acids into proteins nor did actinomycin D influence the effect of insulin to increase protein synthesis The stimulating effect of insulin on the turnover rate of phosphocreatine ATP ADP uridine and guanosine phosphates were unchanged by antinomycin D The findings in the present study that insulin induced glucose uptake and glycogen synthesis were not influenced by actinomycin D (10 μg ml) seem to support the view that insulin effect upon isolated muscle is not mediated through a biosynthesis of messenger RNA

The inhibition of insulin stimulated glycogen synthesis at a higher concentration of actinomycin D (100 μg ml) is at present difficult to explain It might be pointed out

however that at this concentration protein synthesis is strongly inhibited by actinomycin D This inhibition of protein synthesis might in turn interfere with the insulin effect on glycogen synthesis A more marked effect on protein synthesis at higher concentrations of actinomycin D is in accordance with the findings of Reich *et al* (1962)

Two possibilities exist for the inhibition of the insulin induced glycogen synthesis by puromycin. 1) Inhibition of insulin induced synthesis of an enzyme concerned with glycogen synthesis 2) Inhibition of insulin induced reactions leading to increased activity of glycogen synthetase The experiments with actinomycin D at higher concentrations to some extent support the first possibility

Puromycin is known as a specific inhibitor of protein synthesis acting probably as an analogue of esterified transfer RNA (Yarmolinsky and de la Haba 1959) However, it is possible that puromycin as well as actinomycin D are not quite specific in their action, especially at higher concentrations After injection of puromycin on rats a depletion of liver glycogen has been observed (Hofert *et al* 1962) According to Hofert *et al* (1963) glycogenolysis in liver could be produced by puromycin analogues as well. Puromycin aminonucleoside and 6-dimethylaminopurine produced a more marked decrease of hepatic glycogen than puromycin while the comparative degree of inhibition of glycine 2 C incorporation into proteins was reversed A similar glycogenolytic effect of puromycin on muscle has not been observed neither in vivo (Hofert *et al* 1962 nor in vitro (as demonstrated in the present work

In order to study the relationship between the effect of puromycin on protein synthesis and on the insulin induced glycogen synthesis experiments with puromycin analogues are in progress

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however that at this concentration protein synthesis is strongly inhibited by actinomycin D This inhibition of protein synthesis might in turn interfere with the insulin effect on glycogen synthesis A more marked effect on protein synthesis at higher concentrations of actinomycin D is in accordance with the findings of Reich *et al* (1962)

Two possibilities exist for the inhibition of the insulin induced glycogen synthesis by puromycin 1) Inhibition of insulin induced synthesis of an enzyme concerned with glycogen synthesis 2) Inhibition of insulin induced reactions leading to increased activity of glycogen synthetase The experiments with actinomycin D at higher concentrations to some extent support the first possibility

Puromycin is known as a specific inhibitor of protein synthesis acting probably as an analogue of esterified transfer RNA (Yarmolinsky and de la Haba 1959) However it is possible that puromycin as well as actinomycin D are not quite specific in their action especially at higher concentrations After injection of puromycin on rats a depletion of liver glycogen has been observed (Hofert *et al* 1962) According to Hofert *et al* (1963) glycogenolysis in liver could be produced by puromycin analogues as well Puromycin aminonucleoside and 6-dimethylaminopurine produced a more marked decrease of hepatic glycogen than puromycin while the comparative degree of inhibition of glycine 2 ^{14}C incorporation into proteins was reversed A similar glycogenolytic effect of puromycin on muscle has not been observed neither in vivo (Hofert *et al* 1962) nor in vitro (as demonstrated in the present work)

In order to study the relationship between the effect of puromycin on protein synthesis and on the insulin induced glycogen synthesis experiments with puromycin analogues are in progress

Actinomycin D was kindly donated by Dr Mushett Merck Sharp & Dohme The author is indepted to Prof O Walaas for helpful advice and encouragement Thanks are due to Mrs A Adler and Mrs A Eggesvik for technical assistance This work was supported by grants from the Nordisk Insulin Fund and The Norwegian Council for Science and The Humanities

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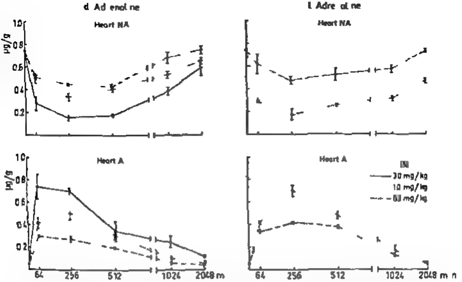

Fig 1 Effect of d and l A on the CA content of rat heart Times on abscissa represent minutes after a single i m injection of indicated dose Each point represents mean of at least 4 determinations (2 tissues pooled for each determination) ± S E M Control values represent mean of 10 saline treated controls NA 0 74 ± 0 05 µg/g A 0 05 ± 0 02 µg/g

free A and NA Three different doses of d A (0 3 1 0 and 3 0 mg/kg) and two doses of l A (0 3 and 1 0 mg/kg) were used Injected amines and the concentration in the tissues and urine are given as the base The volume of the injection was kept constant at all times Rats were sacrificed by a blow on the head and tissues (heart and liver) removed washed with saline blotted weighed and homogenized in 5 trichloracetic acid (TCA) in an Ultra Turrax apparatus Two organ specimens were pooled for each analysis Following homogenization the samples were filtered adsorbed on alumina and CA determinations carried out according to the method of Euler and Lishajko (1961) and read in a Coleman fluorimeter No correction was made for the 10—20 amines lost during the procedure as determined by recovery experiments

For obtaining urine data pooled samples were obtained from 5 rats placed in a community cage These rats were subjected to the same treatment as described above with samples being collected at the same times as for organ analysis plus an additional collection at 4 096 min Collection periods were the intervals between these times A saline treated control group accompanied each experimental group In the case of the urinary studies 0 3 and 1 0 mg/kg d A and 0 1 mg/kg l A were studied In some experiments the fluorimetric data were checked by bioassay using the cat blood pressure and hen rectal caecum (Euler 1956) The fluorimetric and the biologic assay results were in good agreement

Other experiments were carried out in which the heart homogenates were submitted to differential centrifugation and the CA content of the subcellular fractions determined For these experiments 3 hearts were pooled for each determination All procedures conducted following the dissection of the tissues were done at + 2 C The hearts were placed in 30 ml of isotonic potassium phosphate buffer pH 7 5 containing EDTA (2 mg/ml) minced with scissors and homogenized using the Ultra Turrax. This instrument was operated for 15 sec at low speed and 15 sec at high speed A portion of the homogenate was extracted in 10 TCA and used for assay of total heart The remaining homogenate was spun in a Spinco Model L ultracentrifuge at slow speed (8 000 × g) for 15 min. The slow speed supernatant was then spun at high speed (100 000 × g) for 40 min This yielded a supernatant fraction and a sediment which

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From the Department of Physiology Karolinska Institutet Stockholm Sweden

Uptake and Exchange of Catecholamines in Rat Tissues after Administration of d- and l-Adrenaline

By

T C Westfall [1]

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Abstract

Westfall T C *Uptake and exchange of catecholamines in rat tissues after administration of d and l adrenaline* Acta physiol scand 1965 *63* 336—342 — The effect of d and l adrenaline (A) on the catecholamine (CA) content of heart and liver of the rat was studied at various times ing i m administration It was observed that both isomers are readily taken up by these and exchanged for the normal neurotransmitter noradrenaline (NA) The present ments also present evidence for the uptake of both d and l A into subcellular storage granules The fall in the NA content of the organs was accompanied by an increased urinary excretion of NA The present experiments also indicate that there is a slight steric preference for the storage in favor of the l isomer

It has been reported that d adrenaline (A) is capable of producing greater than 95 % depletion of noradrenaline (NA) in various organs of the rat after injection replacing 50—100 % of the lo t NA (Andén and Magnusson 1963) In the present experiments the stereospecificity of this exchange was investigated by studying the effect of the d and l isomers of A on the catecholamine (CA) content in heart and liver of the rat Urinary CA levels were also studied in order to determine if the loss of NA in organs was accompanied by an increased NA excretion Other experiments were carried out in which the effect of d and l A on the CA content of subcellular fractions of rat heart was studied to find out whether these amines could be taken up or exchanged with the NA in the storage granules

Methods

Male rats from the Sprague Dawley strain weighing from 250—320 g were used throughout this study The rats were injected i m with either d or l A and hearts and livers removed at various times (64 256 512 1 024 and 2 048 min) following administration and analyzed for

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difference in action between the same dose of d and l A the l isomer occurring in greater amounts in the granules Even though l A seems to be taken up to a greater extent there appears to be about the same amount of decrease in the NA levels produced by the two forms

Discussion

The present experiments show that both d and l A can be readily taken up by the heart and liver and exchanged for the normal neurotransmitter These results confirm and extend those reported by Andén and Magnusson (1963) Since the above authors did not mention what dose of d A they used it is hard to compare these present results directly with theirs In the present experiments however even with the largest dose of d A used (3 mg/kg) the greatest depletion did not exceed 80 % of the control occurring 256 min following administration

Raab and Gigee (1955) reported that the injection of A resulted in an increase of both A and NA in the heart The present results show however that the A is taken up into the heart in exchange for the NA resulting in a considerable decrease in the NA levels Our results are consistent with those of Stromblad and Nickerson (1961) who also reported that l A could be taken up by heart tissue with a resultant decrease in NA levels

Blakeley *et al* (1964) and Stjarne (1964) have recently emphasized the importance of a recapture mechanism in such organs as the heart for the purpose of maintaining neurotransmitter economy In the present experiments it is felt that the presence of large quantities of d and l A in the area of the axonal membrane results in their being taken up in competition with the recapture of NA which is continually being released from the nerve endings Our experiments also present evidence for the uptake of both d and l A into storage granules This result is in keeping with the report that A can be taken up from the circulation into the sympathetic nerve endings and discharged by nerve stimulation (Rosell and Sedvall 1961 Rosell Axelrod and Kopin 1964)

The present results also show an increase in the urinary excretion of NA following the administration of d and l A This suggests either that the NA is being released from the nerve terminals in larger quantities or that the recapture mechanism is less efficient

As already mentioned the question of the stereospecificity of the uptake of d and l NA is quite controversial Kopin and Bridgers (1963) reported that both d and l NA H^3 were bound in the tissues to the same extent while the rate of disappearance of d NA H^3 was more rapid than that of the l isomer Crout (1964) has recently also observed that both d and l NA H^3 are taken up equally by the rat heart and retained at least for one hour Muller and Shideman (1964) have recently studied the steric preference for uptake of the two isomers of NA by a particulate fraction (105 000 × g sediment) of cat atria They report a limited storage capacity of this fraction and that it exhibits no stereospecificity In contrast to the above results Beaven and Maickel (1964) and Maickel Beaven and Brodie (1963) reported that NA is taken up by a process that is essentially stereospecific for the l isomer Euler and Lishajko (1964) observed that the ATP-dependent uptake in isolated nerve granules of NA and A at low amine concentrations is to some extent stereospecific while the amine uptake at higher concentrations with and without ATP is not or only slightly dependent on the steric configuration Working with an isolated perfused rat heart Iversen (1963) has also reported that at low concentrations the d isomer is taken up at lower rate than the

l isomer however at high concentration the rate of uptake of the d isomer was greater than that for the l isomer at the same concentration Even at the same rate of uptake the resulting tissue concentration of the d isomer may be lower than that of the l isomer if the latter is better retained (cf Kopin and Bridgers 1963)

The present experiments indicate that there is a definite steric preference in favor of the l isomer of A regarding storage into whole tissue as well as in the storage granules

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From the Laboratory for the Theory of Gymnastics, Zoophysiological Laboratory A, University of Copenhagen, and Gentofte County Hospital, Denmark

On the Nervous Factors Controlling Respiration and Circulation during Exercise

Experiments with curarization

By

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Abstract

ASMUSSEN, E., S. H. JOHANSEN, M. JØRGENSEN and M. NIELSEN. *On the nervous factors controlling respiration and circulation during exercise.* Acta physiol. scand. 1965. 63. 343—350. — In the steady state of work on a bicycle ergometer repeated i.v. injections of (+) tubocurarine produced a decrease in handgrip strength. In spite of this the intensity of work was maintained constant for 20 to 30 min. During this time pulse rate and blood pressure increased slightly and irregularly but the ventilation increased, both absolutely and in relation to the oxygen uptake, by up to about 50 %. The cardiac output was uninfluenced by the curarization. By adding CO_2 to the inspired air the alveolar P_{CO_2} was maintained at the normal exercise level. It is therefore assumed that all the known humoral factors controlling respiration in exercise must have been normal. The greatly increased ventilation must consequently have been caused by some nervous factors. The origin of these factors, whether central or peripheral, is discussed on the background of earlier experimental findings. A tentative explanation, based on the assumption that bicycle work is performed by the mediation of the gamma loop, is suggested. According to this the "nervous factor" of respiratory regulation in exercise may be the feed back to the reticular formation of afferent impulses from the muscle spindles.

In recent experiments (Asmussen and Nielsen 1963), employing blocking of the circulation to the working muscles of exercising subjects, it was shown that powerful nervous factors influencing the respiratory centre were operating during the period in which the circulation was blocked. These factors could be explained by assuming the existence of peripheral chemo-receptors in the muscles, where the conditions during the blocking period must become increasingly anaerobic. But the possibility also existed that muscular mechano-receptors were increasingly stimulated during the blocking period, viz. if it was assumed that the gamma loop with its afferents played a major part in the stimulation of the exercising muscles (cf. Granit 1955). It remained un-

explained if such peripheral impulses were also active under conditions where muscular anaerobiosis is absent i e in the normal steady state of light and moderate work

With the intention of exploring this possibility a series of experiments have been performed in which the working muscles were partly paralyzed by means of (+)tubocurarine injected intravenously during uninterrupted work A short preliminary note on these experiments has been published (Asmussen *et al* 1963)

In a study on the effect of (+)tubocurarine on the isometric muscle strength Johansen *et al* (1964) observed that the isometric strength of limb and neck muscles could be reduced to very low values (5—10 of normal) without the respiratory muscles being much weakened as judged from measurements of the maximum voluntary respiratory flow volumes and expiratory pressures This observation opened the possibility of reducing the strength of the leg muscles during bicycling to the lowest value compatible with the continuation of exercise without interfering noticeably with the working capacity of the respiratory muscles The increased motor nerve activity which presumably must be present when a certain part of the muscle fibres are blocked by (+)tubocurarine and the work intensity is constant could thus be studied under circumstances of a normal milieu interieur in the muscles and its influence on respiration and circulation measured

Experiments of a similar character have earlier been performed by Ochwadt *et al* (1959) but their studies were restricted mainly to the initial part of a work period no attempt at reaching a steady state was made and there was no control of the degree of curarization They found that the initial increase in ventilation on beginning a bout of exercise was higher during curarization than in normal condition

Methods and procedure

The bulk of the experiments were performed on two apparently healthy young students They worked after a metronome on a modified Krogh bicycle ergometer in which the saddle had been exchanged for a chair Their feet were tied to the pedals in order to eliminate the danger of loosing the foothold during the period of curarization Their arms were free for measurements of blood pressure, counting of pulse rate and injection of (+) tubocurarine Expired air was collected in Douglas bags through mouthpiece and valve The respiratory movements were recorded on an ink writing recorder by means of a strain gauge arrangement together with the opening and closing times of the taps of the bags When CO_2 mixtures were used as inspiratory air these were administered through a mixing bag which could be kept at an approximately constant volume by adjusting the flow from two cylinders one containing air the other 6 CO_2 in air by means of two flow meters Samples of the inspired air were taken from the tube connecting the mixing bag with the inspiratory side of the valve The volume of the expired air was measured to 0 1 l by emptying the bags by constant suction into a large spirometer and the gas analyses were made on the Scholander gas analyzer The average composition of the alveolar air was determined by means of Bohr s formula using a standard curve for physiological dead space published earlier (Asmussen and Nielsen 195) In some experiments the cardiac output was measured by the acetylene method The (+) tubocurarine was given in small doses through an indwelling needle into a vein on the back of the right hand In order to control the degree of curarization the strength of the handgrip in the left hand was measured at intervals by means of a Collin-dynamometer

Before an experiment the subject rested for about half an hour in the reclining seat of the ergometer During this time his pulse rate was counted repeatedly and his hand grip strength measured He then started work at a constant rate and after about 15 min when he was considered in a steady state two Douglas bags were filled with expired air and control values of handgrip strength pulse rate and arterial blood pressure obtained The injection cannula was then put into place and the first injection of (+)tubocurarine given The first noticeable effect of the curare was always a ptosis of the eyelids that developed quite early after 20

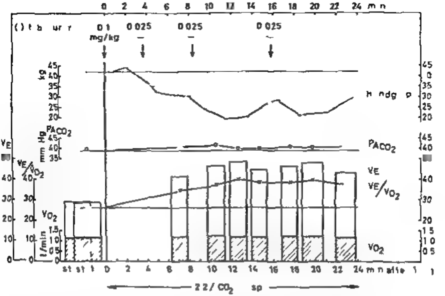


Fig 1 Subj I 380 kpm/min Respiratory functions and handgrip strength in the steady state of exercise before and during curarization

30 sec Subject very the sight became slightly blurred because of eye muscle paresis After 3—5 min a decrease in hand grip was apparent and shortly thereafter a second dose of curare was given By frequent measurements of hand grip strength and repeated graded injections of curare the hand grip strength could be adjusted to a fairly constant percentage of the control value and maintained there for the rest of the experimental period When a reasonable decrease in strength had been obtained repeated collections of expired air began At the same time the CO_2-content of the inspired air was raised to an empirically determined value that would suffice to keep the alveolar P_{CO_2} at the normal exercise level The subject continued working after the metronome Subjectively the work was felt as becoming heavier On occasions when too much curare had been given it was impossible for the subject to keep up the rhythm He was urged to do his utmost and after a few minutes he again recovered strength enough to continue Over a period of 20 to 30 min of continuous work under influence of curare expired air was collected in 6 to 8 Douglas bags each sampling period lasting 1.5 to 2 min In the intervals counts of pulse rate and measurements of blood pressure and hand grip strength were made In special experiments the cardiac output was determined both while working in the normal condition and during curarization In others electromyograms were recorded via plate electrodes pasted to the skin over the lateral vastus muscle

At the stop of the experiments 1 mg of atropine was injected i v and a few minutes later 1—1.5 mg of prostigmine was given The effect of curarization was quickly eliminated in this way handgrip strength returned to normal values and the ptosis disappeared Subjectively feeling of heaviness with some clumsiness of movements persisted for some hours afterwards

Control experiments In order to make sure that (+) tubocurarine in the concentrations and amounts given had no direct effect on respiration and circulation experiments were performed in the resting state It was found that asde from the decrease in muscular strength curarization had no effect on the functions studied Ventilation oxygen uptake alveolar P_{CO_2} pulse rate and arterial blood pressure remained unchanged during the half hour period of curarization Further work experiments with the same intensity and duration of work as in the curare experiments but without curarization the functions studied (muscle strength ventilation oxygen uptake pulse rate and blood pressure) remained in practically steady state with but slight changes attributable to developing fatigue

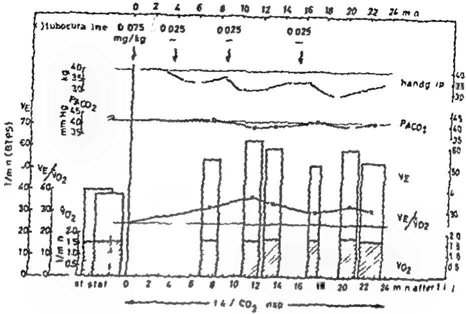


Fig 2 Subj M 713 kpm/min. Respiratory functions and handgrip strength in the steady state of exercise before and during curarization

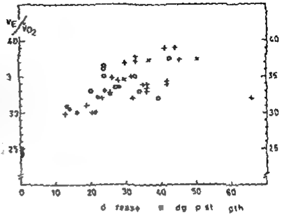
Results

Respiratory functions More than 20 expts on 2 young male subjects with work intensities of 380 kpm/min or 713 kpm/min and with 20 to 30 min of work under the influence of (+)tubocurarine were performed In principle the results were identical and the two examples presented in Fig 1 and 2 are representative for the whole series

Fig 1 shows the results with subj I working at 380 kpm/min It can be seen that soon after the injection of the first dose of curare (0 1 mg/kg) the hand grip strength decreased from its normal value of about 43 kg and by repeating the injections it was maintained at values between 20 and 30 kg for the following 24 min The oxygen uptakes increased slightly presumably due to increasing difficulties in maintaining the working position in the seat of the ergometer The increase was of the order of magnitude of 10 % The ventilation was considerably increased from about ?? l/min (BTPS) to around 45 l/min i e an increase of about 50 Expressed as ventilation pr l O uptake the ventilation increased from about 26 l litre O_2 to about 38 l/litre O_2 i e also an increase of close to 50 By adding 2 2 CO_2 to the inspired air during this period of relative hyperventilation the alveolar P_{CO_2} was maintained close to the steady state value of 39 mm Hg the highest deviation being 2 mm Hg (during the second sampling period)

Fig 2 shows the corresponding results from an experiment with subj M working at 713 kpm/min His hand-grip strength was reduced from 40 kg to values between 30 to 35 kg Attempts to reduce it further disclosed that he was actually working at the limit of his capacity with this reduction in hand grip strength In the short period between 18th and 19th minute when his hand-grip strength was at the lowest 29 kg he was

Fig 3 Pulmonary ventilation (l BTPS) per liter oxygen uptake (STPD) in relation to per cent decrease in handgrip strength Black rectangle normal condition of exercise subj I and M working at 380 kpm/min or 713 kpm/min Open circles and lying crosses subj I working at 713 and 380 kpm/min respectively Black dots and upright crosses subj M working at 713 and 380 kpm/min respectively



unable to keep time with the metronome The oxygen uptake was 1 6 l/min in the normal steady state of work and increased maximally to 1 8 l/min probably for the same reasons as mentioned before This corresponds to a 12 5 % increase The ventilation which normally was about 40 l/min (BTPS) increased maximally to 63 l/min i e by 57 % The ventilation per liter O_2 uptake increased from 24 l/litre O_2 to maximally 37 l/litre O_2 i e by 54 P_{ACO_2} was in this experiment 42 mm Hg in the normal state By addition of 1 4 % CO_2 to the inspired air it was maintained approximately constant The largest deviation (second sampling period) was —2 7 mm Hg

Inspection of curves corresponding to the ones presented as Fig 1 and 2 leaves the impression that the degree of relative hyperventilation depends on the degree of curarization as expressed in the decrease of hand grip strength This impression is strengthened by the fairly good correlation found in all experiments between relative ventilation ($\dot{V}_E/\dot{V}_{O_2}$) and percentage decrease in hand grip strength as seen in Fig 3

Circulatory functions The pulse rate and the arterial systolic blood pressure were measured at intervals throughout the whole work period Both functions increased somewhat during the period of curarization more in subj I than in subj M In the 2 expts presented in Fig 1 and 2 the pulse rate increased from about 120 beats/min to about 130 beats/min in Fig 1 and from 138 beats/min to about 160 in Fig 2 Arterial blood pressure rose from 130 mm Hg to 140 mm Hg in Fig 1 but remained practically constant around 130 in Fig 2 The cardiac output was determined in special experiments on subj I only working at 713 kpm/min The mean values from 5 determinations without and 4 determinations with curarization are shown in Table I

Table 1

	$\dot{V}_{O_2}$ (l/min)	(a-v̄)O_2-diff (ml/l)	Q (l/min)
normal	1 76	102 (87—109)	17 3
curarized	1 87	106 (93—114)	17 7

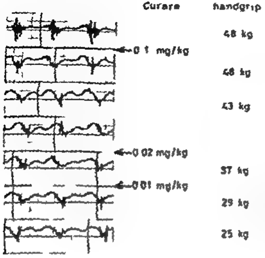


Fig. 4. EMG's from m. vastus lateralis of subj. I during bicycling at 713 kpm/min, 60 revolutions/min. Skin electrodes, ink recorder. Top recording: normal conditions of work. Following recordings were made 4 to 18 min after first injection of (+)tubocurarine as shown to the left. To the right the injections of curare and the measured handgrip strenghts are indicated at the approximate times.

It can be seen that the cardiac output is only insignificantly influenced by the curarization.

Electromyography. In Fig. 4 are shown some emg.'s recorded via skin electrodes and an inkwriter during bicycling in the normal state and at different times during the period of curarization. The most noteworthy effect is that the summated emg.'s become smaller with increasing degree of curarization in spite of the fact that work proceeded at a constant rate.

Discussion

The present experiments with curarization during continuous steady state exercise showed that the pulmonary ventilation increased considerably both absolutely and in relation to the oxygen uptake. The relative increase was — with the uncertainties inherent in the method of judging the degree of curarization from the strength of the hand grip — proportional to the degree of curarization.

It seems justifiable to assume that with the adjusted constancy of $P_{A_{CO_2}}$ the humoral factors known to influence respiration were the same in the curarized condition as in the normal state of constant exercise. At the light intensity of exercise in the normal condition there is presumably no accumulation of lactic acid or other acid products and as the cardiac output is uninfluenced and local blood flow unimpeded there seems no reason to assume that any change in $[H^+]$ should occur during the period of curarization. Neither is there any reason for assuming that other anaerobic metabolites should occur in increased amounts during the period of curarization.

The recorded increase in ventilation therefore must be due to some nervous factor operating with increased intensity during the curarization period or called forth directly by the altered conditions of work. Such nervous factors may be either of peripheral or of central origin. In the former case they might originate in receptors in the working muscles, e. g. in mechano-receptors, thermo-receptors, chemoreceptors, pain receptors etc. In the present experiments, where the intensity of work was maintained constant

the direct stimulation of the mechano-receptors in muscles tendons joint surfaces etc caused by the muscular contractions and the movements must have been the same with and without curarization Neither is there any reason to assume that the other receptors are stimulated differently in the two situations Therefore a direct increase in afferent impulses from the moving parts is hardly to be assumed

On the other hand a considerable increase in the efferent flow of nervous impulses to the curarized muscles must be assumed If curare blocks the motor endplates of a certain number of individual muscle fibres in the active motor units the flow of motor impulses to the muscles must be increased in order to maintain the constant work This can be thought to occur either by an increased frequency of stimulation or by recruitment of new motor units or by both The situation to a certain degree resembles that obtaining during exercise with blocked circulation (Asmussen and Nielsen 1963) Only with curare the output of tension per active motor unit is decreased because some of the fibres in the units are blocked at the endplate whereas in the experiments with blocked circulation the output of tension per motor unit is decreased because all the fibres in the unit become fatigued due to the anaerobic condition. The emg findings seem to substantiate this In the experiments with blocked circulation the electrical activity picked up by plate electrodes over the working muscles showed an increase with time (Asmussen and Nielsen 1963) whereas in the present experiments it decreased (Fig 4) possibly because the syncronized electrical output per motor unit decreased as the individual muscle fibres became curarized (cf Locke and Hennemann 1960)

In the present experiments and in our earlier experiments with blocked circulation there seems to exist a positive correlation between the efferent motor out flow and the ventilation An obvious explanation of this fact would be to assume that the nervous impulses from the motor cortex on their way down through the brain stem influenced the respiratory center e g by irradiation as suggested by Krogh and Lindhard (1913) or in accordance with Burns (1963) by increasing the surrounding traffic of activity in non respiratory parts of the reticular formation The first and simplest of these explanations is contradicted by the findings that electrical stimulation of muscles in conscious man brings about an increase in ventilation that is related to oxygen uptake in exactly the same way as in volitional work (Asmussen Nielsen and Wieth Pedersen 1943) as well as by the findings of Kao (1963) who in cross-circulation experiments on dogs found that peripheral nervous impulses from exercising muscles stimulated the respiration Both these works pointed to the importance of afferent nervous impulses for the regulation of respiration in exercise

An increased afferent flow of impulses to the respiratory centre or its surroundings can however be postulated also in the present experiments even though the outer work remains constant if one assumes that the stimulation of the lower motor centres wholely or dominantly takes place via the gamma loop cf Granit 1955) In that case the need to expand the motor activity to more units would primarily be met by an increased flow of impulses to the intrafusal muscle fibres of the muscle spindles which in turn would produce an increased activity in the afferents to the large moto-neurons in the medulla and presumably an increased feedback to the centres in the reticular formation

Also other kinds of information related to the work going on must be assumed to reach the brain stem e g from the acoustic and optic centres where the beat of the metronome the movements of the pedals etc are signalled It may well be that it is the sum total of all these signals impinging on the cells in the formatio reticularis surround

ing the respiratory neurons that constitute the nervous factor in respiratory regulation during exercise At our present state of knowledge it seems impossible to locate this factor more precisely but the present experiments speak in favour of its existence

The circulatory functions during work with curarization are much less influenced than in the experiments with blocked circulation (Asmussen and Nielsen 1963) Both blood pressure and pulse rate increased much less than in the blocking experiments whereas the cardiac output remains uninfluenced as in the earlier experiments The reason for the difference between these and the earlier experiments may be sought in the local conditions in the muscles In the present experiments normal aerobic conditions must have prevailed whereas in the former experiments with blocked circulation, a state of increasing anaerobiosis existed The possible effect of impulses from peripheral chemo-receptors (Stegeman 1963) must have been quite different in the two cases The small changes observed during curarization may have been caused by an increased outflow of adrenalin as the work subjectively was felt more strenuous

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Acta physiol scand 1965 63 351—357

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Some Quantitative Considerations on Vagally Induced Relaxation of the Gastric Smooth Muscle in the Cat

By

G Jansson and J Martinson

Rece ved 6 July 1964

Abstract

Jansson G and J Martinson *S me quant tatie considerations on v ally induced relaxation of the gast c smooth muscle in the cat* Acta physiol scand 1965 *63* 351—357 — The vagus nerves of the cat contain two sets of efferent fibres affect ng gastric motility These two sets can be distinguished from one another by the d fference in their st mulat on threshold The low threshold fibres el cit pure motor responses the h gh threshold ones a long lasting relaxat on of th stomach wall Th s vagally induced gastric relaxation which seems to be confined largely to the corpus and fundus may somet mes be extreme When recording gastric vol me changes at a constant low transmural pressure (2—3 cm H_2O) stimulation of the inhib tory fibres can increase the volume several hundred per cent Under these conditions the fundal muscle fil res may increase by 40 or more in length Essent ally max mal relaxation of the stomach wall can apparently be brought about by stimulation of these inhib tory fibres It would appear that th s powerful relaxing effect of the vagal fibres on gastric muscle is invol ed in the "recepti e rel xation of the stoma h

The effect of vagal stimulation on gastric motility received wide attention during the first decades of the century (for review see McSwiney 1931) It is known that in certain circumstances vagal stimulation inhibits gastric motility but the underlying mechanism and the physiological significance of such inhibition has been a matter of dispute

In a recent study on cats Martinson and Muren (1963) who used graded efferent st mulation of the vagus nerves obtained results suggesting the existence of two types of efferent vagal fibres to the stomach a low threshold one causing excitatory responses and a high threshold one causing relaxation of the stomach wall The gastric motor inhibition induced by the high threshold fibres is not significantly affected by atropine given in doses suffici nt to block the excitatory responses (*cf* Harrison and McSwiney 1936) As to the peripheral mechanism eliciting the inhibition Greeff Kasperat and Osswald (1962) and Paton and Vane (1963) have independentl produced evidence that it is mediated by an adrenergic mechanism

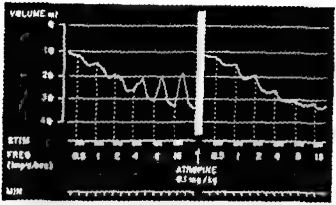


Fig. 1. Cat 2.5 kg. Gastric volume recorded at constant transmural pressure 3 cm H_2O. Stimulation with varying impulse frequencies, pulse duration 2 msec, 5 V. Atropine administered i.v.

The present study is concerned with the degree of influence of the vagal inhibitory fibres on gastric motility with special reference to their capacity to augment the volume of the stomach.

Materials and methods

The material consisted of 29 cats weighing between 1.5 and 3.9 kg. The animals were deprived of food for 24–36 hours before use. The animals were anaesthetized with ether followed by chloralose (50–80 mg per kg b. w.) given i.v. A tracheal cannula was inserted to secure a free air passage.

The vagus nerves were dissected free in the neck region and divided. The peripheral ends were placed on bipolar silver electrodes in such a way as to prevent drying and cooling of the nerves. In two experiments that part of the anterior vagal trunk below the diaphragm was prepared for stimulation in the same way as the nerves in region of the neck. The electrodes were connected to a Grass Mod. S 4 stimulator producing square waves pulses of adjustable intensity, duration and frequency.

The abdominal cavity was opened by a midline incision. In most of the experiments the intestines except the oralmost part of the duodenum were removed. The stomach was isolated by ligation of the cardia in such a way as not to damage the vagal nerve trunks which passed undamaged outside the ligature. A wide bore plastic catheter was introduced into the stomach via the pylorus where it was held in position by means of a ligature around the pylorus and then connected to a volume reservoir fitted with a recording device. The volume reservoir was filled with isotonic saline and arranged in such a way that the intragastric pressure could be set at desired level. The temperature of the saline was kept at about 38° C.

When it was desired to ascertain the exact transmural pressures applied across the stomach wall (e. g. for experiments illustrated in Fig. 3) the open abdominal cavity was filled with Tyrode's solution and the whole stomach thereby immersed in situ in fluid kept at 38° C. The transmural pressures applied were deduced from the differences between the fluid level of the reservoir and that in the abdominal cavity.

In nine experiments the opening in the abdominal wall was covered with a perspex lid which was sutured to the surrounding tissue. This device thus provided a window allowing direct inspection of the stomach.

Most of the time during an experiment the pressure was set at zero and the stomach was then empty. Separated by an interval of at least 15–20 min each experimental event was

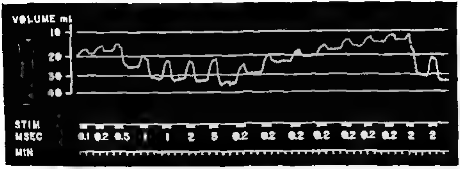


Fig 2 Cat 2 5 kg Gastric volume recorded at a constant transmural pressure 3.5 cm H_2O Stimulation with constant impulse frequency 8 imp/sec 5 V varying impulse duration according to scale (msec)

started by establishment of the desired transmural pressure This procedure proved to give the most readily reproducible initial volumes and responses to vagal stimulation

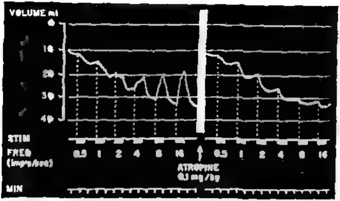
Vagal stimulation was performed in two ways according to the grade of the stimuli used The first type of stimulation was considered to activate all efferent vagus nerve fibres. This means that at a voltage of 5 the impulse duration was 2 or sometimes 5 msec In these experiments the pulse frequency was varied to grade the responses (Fig 1) The other type of experiment utilized the technique described by Martinson and Muren (1963) to distinguish between excitatory and inhibitory nerve fibres in the vagus nerves Frequency and impulse voltage were kept constant and pulse duration varied according to the scale 0 05 0 1 0 2 0 5 1 msec An experiment of this latter type is illustrated in Fig 2

Results

Stimulation of all efferent vagal fibres produced a characteristic response (Fig 1) Gastric volume was recorded at a constant transmural pressure of 3 cm of water and the vagus nerves were stimulated with increasing pulse frequencies The left hand panel of Fig 1 shows that both excitatory and inhibitory effects could be elicited During the short phase of stimulation the response was dependent on the initial volume When the initial volume was small and the stomach at rest the stomach wall reacted with relaxation whereas when the volume was increased an initial contraction was seen especially when a relaxation had been brought about by a preceding stimulation of the inhibitory vagal fibres The contractions observed during stimulation ceased as soon as stimulation was stopped, but the stomach continued to be relaxed for a relatively long time This relaxation follows a frequency response relationship (*cf* Martinson 1964)

It is clear from Fig 1 that the after relaxation was not a result of the contractions *per se* since the excitatory responses were abolished by a small dose (0 1 mg/kg) of atropine Note that in this case the largest volume of the stomach recorded during stimulation was exactly the same as that noted during the after relaxation

Fig 2 illustrates an experiment where the stimulation characteristics were varied to permit stimulation of the excitatory and inhibitory fibres influencing gastric motility (Martinson and Muren 1963) Stimulation of the excitatory low threshold fibres (4 V 0 1 and 0 2 msec) produced a true reduction of volume (increase of tonus) As



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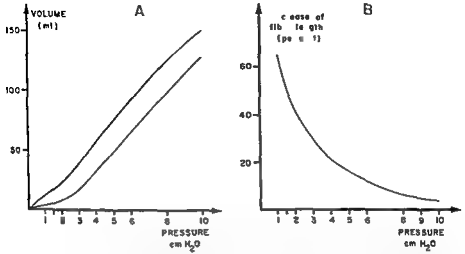

Fig 4 4 Pressure volume diagram of resting stomach (lower curve) and of stomach relaxed by vagal stimulation 8 imp/sec 2 msec and 5 V (upper curve) Mean values from 4 expts similar to that illustrated in Fig 3 *B* Increase in fibre length induced by vagal stimulation at different transmural pressures Based on calculation of cubic roots of volumes in section A For further explanation see text

pressure range At levels above 3—4 cm H_2O however the stomach relaxed with a larger increase in volume per unit of pressure

After vagal stimulation (8 imp/sec 2 msec and 5 V) gastric volume increased by almost the same amount over the entire range used This means that the relative increase in volume was largest in the low pressure range For instance in the experiment illustrated in Fig 3 the volume was doubled at transmural pressures below 4 cm H_2O but increased by only about 15 % at 8 cm H_2O In other experiments the vagally induced relaxation produced a more than 5-fold increase in volume in the low pressure range In 13 expts stimulation of the vagal inhibitory fibres increased the gastric volume from on the average 12 ml to 35 ml *i e* 3 fold at a transmural pressure of 3 cm H_2O The pressure volume diagram for the vagally relaxed stomach has the same general appearance as that for the resting stomach but the bend at 3—4 cm H_2O is less prominent (Fig 4 A upper curve)

Fig 4 A can be used to estimate the length of the single smooth muscle fibres of the fundus Assuming that the fundus is approximately spherical the cube root of the intragastric volume will give an equally approximate measure of the length of the fibres Since a sphere encloses more volume per unit of surface than any other body such approximation will at any rate not overestimate fibre length. In Fig 4 B the vagally induced relaxation of fundal smooth muscle fibres is given in terms of increase of fibre length at different transmural pressures In the pressure range below 3 cm H_2O the vagal inhibitory fibres can relax the single fibres by about 40 % or more in the higher pressure range when the fundus is already relaxed the increase in length is less than 10

Discussion

Stimulation of peripheral end of the vagal nerves in the neck will activate both excitatory and inhibitory nerve fibres to the stomach provided the intensity of the stimulation pulses is high enough (Martinson and Muren 1963) Stimulation of the inhibitory fibres produces relatively prolonged relaxation of the stomach muscle cells This relaxation outlasts by *far the response of the excitatory fibres to stimulation* This is clear from Fig 1 A small dose of atropine abolished the excitatory but not the inhibitory responses (right panel) and the relaxation during stimulation was the same as the after relaxations (left panel)

Textbook articles on the effect of vagal stimulation on gastric motility are based on early work by McSwiney and Wadge (1928) who found that the effect of vagal stimulation depends on the tonus of the stomach, when the tone is low vagal stimulation will cause contraction when high relaxation Fig 1 and 2 show records giving support to this view provided the stimuli employed are intense enough to activate both the excitatory and inhibitory fibres When however only excitatory fibres are stimulated the response will be only excitatory irrespective of the initial state of tone (see Fig 2 effect of stimulation with pulse duration 0 2 msec) Although McSwiney and Wadge's experiments were intended to evaluate the effect of different stimulation intensities it now seems safe to assume that the stimuli they used were such as produced a mixed response *i e* of both excitatory and inhibitory vagal fibres

The prolonged inhibitory response illustrated in Fig 2 suggests that the vagal fibres either initiate a reverberating activity on local neurones or perhaps and possibly more likely that they release some very stable smooth muscle relaxing substance that is not easily removed by the blood stream

The very strong and differentiated action of the vagal inhibitory fibres affecting the region of the corpus and fundus but apparently not that of the pyloric antrum suggest that they may be of considerable physiological importance though no *definite* information is available concerning the circumstances in which they are normally activated The fibres are most potent in a low transmural pressure range when the stomach contains only a small volume Available data (*e g* Cannon and Lieb 1911 Hesser and Perret 1960) indicate that the pressure within the resting stomach of the normally conscious animal is low *i e* in the order of 5 cm H_2O or less The transmural pressure is then probably somewhat lower and this means that the pressures used in the present study are well within the physiological range *Furthermore the stomach* is known to have a remarkable capacity to relax when filled a phenomenon called *receptive relaxation* (Cannon and Lieb 1911) This phenomenon is said to be confined mainly to the fundus (Lind Duthie Schlegel and Code 1961) Though much attention has been paid to this function little is so far known about the exact mechanisms involved

When the resting acutely decentralized stomach is subjected *to pressures above* 3—4 cm water *it will relax more per unit pressure increase* than when subjected to corresponding increases in a lower pressure range (Fig 4) Paton and Vane (1963) observed a similar phenomenon on the isolated stomach and termed it fundal release because it was seen only in the fundus of the stomach This might be taken as an indication that local reflex systems are mainly responsible for the receptive *relaxation* However several observations indicate that the receptive relaxation may be part of the *cephalic phase* of digestion Thus Cannon and Lieb (1911) noted that the

relaxation can be abolished by vagotomy. Olbe and Jacobsson (1963) found a fairly long inhibition of type II waves in the stomach during sham feeding. Inhibitory gastric motor responses mediated by the vagus nerves and originating in different central nervous structures have been described by Eliasson (1952 and 1954) and Hesser and Perret (1960) who however said nothing about the degree or localization within the stomach of these centrally induced gastric relaxations. A reflex mechanism involving vagally mediated relaxation of the type described may be expected to have an afferent link originating in receptors in the upper digestive tract and in the stomach itself. The general response of the stomach to stimulation of afferent vagal fibres is a marked relaxation which in degree and duration strikingly resembles the relaxation noted in the present study (Harper, Kidd and Scratcherd 1959). It seems reasonable to assume that the vagal inhibitory fibres studied in the present investigation may form the efferent link for such reflex and centrally induced "receptive relaxations" of the stomach.

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Vagal Release of Gastrin in Cats Following Reserpine[1]

By

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Abstract

EMÅS, S. and B. FYRO. *Vagal release of gastrin in cats following reserpine.* Acta physiol. scand. 1965. 63. 358—369. — Reserpine treatment of nonanesthetized cats has previously been shown to sensitize the HCl-secreting cells to injected gastrin, probably by a centrally induced enhancement of vagal tone. Indications were obtained also of an interference with the gastrin mechanism. Since vagal impulses are considered to be able to release gastrin, the effect of reserpine on the gastrin activity of the antral and duodenal mucosa has been investigated. Gastrin was extracted from the mucosae of reserpine injected and untreated cats and the secretory activity of the preparations determined on nonanesthetized gastric fistula cats. An intramuscular injection of 0.10 mg of reserpine per kg b. w. reduced the gastrin activity of the antral mucosa by about 50 per cent in 24 hrs. Neither a 20-fold increase in dose nor repetition of the injection of 0.10 mg per kg every 24 hrs for 2 or 4 days produced any further reduction and the gastrin activity returned to normal level despite repeated injections for 8 days. An intravenous infusion of 0.03 mg of reserpine per kg, which produces gastric hypersecretion of acid for several hours, caused no significant alteration in the gastrin activity of the antral mucosa. The gastrin activity of the duodenal mucosa amounted to about one tenth the activity of the antral mucosa and was reduced by 0.10 mg of reserpine i. m. daily for 2 or 4 days. Bilateral vagotomy did not alter the gastrin activity of either the antral or duodenal mucosa nor did the i. m. injection of 0.10 mg of reserpine per kg daily for 2 days in vagotomized cats. It is suggested that reserpine causes release of antral and duodenal gastrin by central vagal activation. The significance of gastrin release as a mechanism by which reserpine activates the HCl-secreting cells is discussed.

There is good evidence that cholinergic drugs (Langlois and Grossman [illegible], Burstall and Schofield 1954) and vagal impulses (Uvnäs 1942, Maung Pe Thein and Schofield 1959) elicit gastric acid secretion partly by released gastrin. Reserpine, 0.10 to 0.15 mg per kg b. w. i. m., injected every 24 hrs for 3 days or more increased the acid secretory response of nonanesthetized cats to exogenous gastrin (Emås 1963), probably by enhancing the vagal tone (Emås 1964). However, reserpine treatment did not increase the secretory response to a cholinergic drug or to insulin hypoglycemia (Emås 1963). Possible reasons for this were previously mentioned (Emås 1964). One conceivable explanation could be that smaller amounts of gastrin were available for release by the cholinergic drug or by vagal impulses in reserpine treated than in untreated cats.

[1] Parts of this investigation were presented at the Second International P[illegible] [illegible]ical Meeting in Prague, Czechoslovakia 1963 (Emås and Fyro 1963).

To elucidate whether reserpine reduces gastrin activity of the antral mucosa, gastrin was extracted from this part of the stomach of reserpine injected and control cats, and the secretory activity of the preparations determined on nonanesthetized gastric fistula cats by a previously developed technique (Uvnäs and Emås 1961). A gastrin-like activity has been demonstrated in the duodenal mucosa of dogs (Komarov 1938, 1942), hogs (Harper 1946) and humans (Uvnäs 1945) and a few extracts were therefore prepared also from the mucosa of the proximal duodenum.

Reserpine was found to reduce the gastrin activity of the antral and duodenal mucosa and — since evidence has been presented that reserpine treatment increases vagal tone and vagal impulses release gastrin — this reduction could be due to vagal release of gastrin. This was investigated by extracting gastrin from the antral and duodenal mucosae of vagotomized cats subjected to reserpine treatment and determining the secretory activity of the preparations.

Methods

Healthy cats of both sexes (2.0 to 3.5 kg) were injected with reserpine (Serpedin®) and killed by air embolism 6, 9, 12 or 24 hrs later, or else the reserpine injection was repeated every 24 hrs for 2, 4 or 8 days and the animals killed by air embolism 24 hrs after the last injection. In one series of experiments (Series II, see below) the control cats received the vehicle for Serpedin® which was administered i.m. daily for 4 days. In the other series the control cats were not injected with the vehicle. The stomach and proximal duodenum were removed immediately after death in all series except in Series I in which they were removed during pentobarbital anesthesia (Nembutal®, Abbott, 25 mg per kg of b. w. i. v.). The same results were obtained whether anesthesia was used or not. Animals subjected to 2 or more injections with reserpine were fed with fish twice a day by hand if they did not eat by themselves. No food or water was allowed for 24 hrs before death. Pregnant animals were excluded.

Listed below are 7 series of experiments on nonvagotomized cats. In every series 5 cats were used as controls and reserpine was given to one or more groups of 5 cats also. In Series I to V reserpine was injected in the dose of 0.10 mg per kg of b. w. i. m. Series VI cats received 2.0 mg per kg i. m., and 0.03 mg per kg was administered i. v. to Series VII cats. In the individual series the groups of cats were treated as follows (the descriptions of the bars refer to Fig. 1 to 3).

Series I (bars diagonally shaded). Control; reserpine daily for 4 days.

Series II (white bars). Control (the vehicle for Serpedin® daily for 4 days); reserpine daily for 4 days.

Series III (black bars). Control; one reserpine injection 12 hours before death; one reserpine injection 24 hrs before death; reserpine daily for 2 days; reserpine daily for 8 days.

Series IV (bars diagonally shaded). Control; one reserpine injection 6 hours before death; reserpine daily for 8 days.

Series V (bars with horizontal strokes). Control; one reserpine injection 6 hours before death; one reserpine injection 9 hrs before death.

Series VI (Fig. 4). Control; one i. m. injection of reserpine (2.0 mg per kg of b. w.) 24 hrs before death.

Series VII. Control; one i. v. 15-min infusion of reserpine (0.03 mg per kg of b. w.) 24 hrs before death. Reserpine was diluted in 0.9 per cent NaCl solution immediately before starting the infusion (infusion pump delivering 0.40 ml per min).

Another series of experiments (Series VIII) were performed on nonvagotomized and vagotomized cats. The animals were provided with gastric cannulas (Emås 1960). After recovery (at least 2 weeks) bilateral th[illegible]tomy was performed as previously described (Emås 1964). The vagotomy [illegible] complete in all animals [illegible] insulin hypoglycemia produced no gastric acid secretion despite blood sugar levels of less than 40 mg glucose per 100 ml blood during [illegible] hypoglycemia (Emås 1964). The experiments with insulin were performed [illegible] described [illegible] paper [illegible] 1963. The animals were killed within 2 months after complete vagotomy. [illegible] series 2 groups of 3 cats [illegible] no reserpine and another 2 groups of 3 [illegible] 0.10 mg of reserpine per kg of b. w. [illegible]

Series VIII (Fig. 5). Nonvagotomized control; reserpine to nonvagotomized cats daily for 2 days; vagotomized cats (no reserpine); reserpine to vagotomized cats daily for 2 days.

Preparation of Mucosa Specimens

The stomach and proximal duodenum were opened along the lesser curvature and the mucus gently scraped off from the mucosa. Only empty stomachs were kept.

The antral mucosa was stripped from 2.5—3 cm to 0.5 cm proximal to the pyloric sphincter. In Series I to VII the antral mucosae from each group of 5 cats were divided along the greater curvature in approximately equal halves and randomly assigned either to Set A or B, rapidly frozen and stored at — 20° C until the extraction procedure started within 3 days. Two sets of half antral mucosae from each group of cats made it possible for duplicate estimations of both yield of gastrin preparation and gastrin activity per g of antral mucosa (illustrated by the double bars in Fig. 1 to 4). A total of 38 sets from 19 groups of cats were obtained. In Series VIII the antral mucosae were not divided and duplicate estimations were not made (single bars in Fig. 5). The antral mucosae from each group of 3 cats made a set. Thus since 12 cats were used in this series, 4 sets of mucosae were obtained.

The duodenal mucosa was stripped from a segment of duodenum 4 cm in length with its proximal border 0.5 cm distal to the pyloric sphincter. In Series I the mucosae from each group of cats were divided longitudinally, randomly assigned either to Set A or B and stored as above. Each set was made up of 3 half duodenal mucosae. The total of 4 sets from 2 groups of 3 cats made duplicate estimations possible (see Table I). In Series VIII each set consisted of 3 undivided duodenal mucosae. A total of 4 sets from 4 groups of 3 cats were obtained.

Preparation of Gastrin

After standardization, the method of Komarov (1938) has shown a satisfactory reproducibility in the yield of gastrin preparations in respect of both weight and secretory activity (Emås and Fyro 1964). It was therefore used in the present study.

Gastrin was prepared separately from each set but simultaneously from all sets within a series. Care was taken to treat all batches exactly the same.

One preparation was randomly selected from each series and assayed on guinea pig ileum for histamine activity. No histamine was detectable in any preparation (< 0.01 μg of histamine dihydrochloride per mg of dried preparation).

Assay of Gastrin

The gastrin preparation obtained from each set of antral mucosae was assayed once (twice in Series II) on each of 4 nonanesthetized gastric fistula cats according to Uvnäs and Emås (1961). All preparations within a series were assayed on the same 4 animals. One cat was used in common for the assays in 6, another for those in 7 of the series.

Due to the low secretory activity of the preparations obtained from the duodenal mucosa, the amounts of preparation available in Series I allowed each preparation to be assayed only on 3 cats, once on each animal. All preparations in this series were assayed on the same 3 animals used also in the above mentioned assays of corresponding preparations from the antral mucosae. Similarly, each preparation in Series VIII was assayed on 3 animals used throughout the series.

The assay technique and the estimation of secretory activity of the preparations have been described by Uvnäs and Emås (1961). An approved assay requires in principle that the mean 1 hour secretory response of total acid (in milliequivalents, titrated with phenolphthalein as indicator) to two identical doses of the gastrin preparation should be equivalent to or greater than the response to a preceding, relatively small dose of histamine, without exceeding the response to a subsequent, relatively large histamine dose. The dose of the gastrin preparations was in most assays 0.5 mg per kg of b. w. If 3 mg of a gastrin preparation per kg in two subsequent infusions was insufficient to evoke a mean response equal to or greater than the response to the preceding infusion of histamine (in a dose adjusted to produce about minimum response (Uvnäs and Emås 1961)), the mean response to the preparation was recorded as less than that to histamine (Emås and Fyro 1964). Such a low secretory activity was found only in preparations from the duodenal mucosa of nonvagotomized cats injected with reserpine. All preparations, however, elicited at least a slight acid secretion.

The secretory activity of the preparations was expressed in histamine units (HU) per mg of dried preparation. The secretory activity of a preparation is 1 HU if 1 mg of the preparation per kg of b. w. elicits the same 1 hour secretory response of total acid as 0.001 mg of histamine

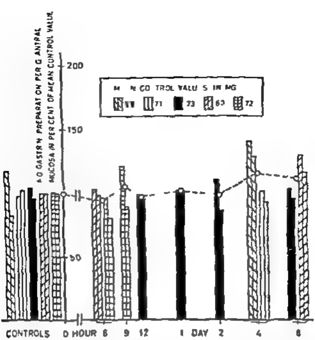

Fig 1 Mean yield of gastrin preparation per g antral mucosa (frozen) of nonvagotomized cats injected with 0 10 mg of reserpine per kg of b w im every 24 hrs in per cent First injection given at 0 Cats receiving a single reserpine injection were killed 6 9 12 or 24 hrs later while cats receiving repeated injections were killed 24 hrs after the last injection Bars with the same marking make a series (Series I to V) Each bar represents the mean yield from 5 half antra of 5 cats (see Methods) The 100 per cent level for each series is the mean yield of preparation in mg per g mucosa from 2 control sets of 5 half antra (boxed data) The broken line connects the figures for mean yield at different times

d hydrochloride per kg of b w both substances infused i v for 15 minutes (Uvnäs and Emås 1961) The HU per g of the mucosa referred to as antral (duodenal) gastrin activity was estimated from the figures for secretory activity of the preparation and for yield of preparation per g of mucosa (frozen)

Evaluation of Data

Experimental data were analyzed in accordance with current methods for analysis of variance the treatment effects in Series I to VII being separated by orthogonal comparisons each with one degree of freedom (Snedecor 1956 Chapt 11)

Results

Gastrin Preparations from the Antral Mucosa of Nonvagotomized Cats

The mean yield of gastrin preparations from control antra varied in Series I to VII between 58 and 74 mg per g frozen mucosa and the mean secretory activity of the control preparations between 17 and 31 HU per mg The mean control values for antral gastrin activity fell between 1000 and 2150 HU per g of mucosa The mean control values in Series II (control cats injected with the vehicle for Serpedin®) fell within the range of those in Series I and III to VII untreated control cats) It was therefore

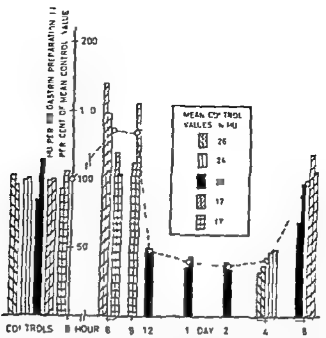

Fig 2 Mean secretory activity per mg gastrin preparation from the antral mucosae of non vagotomized cats injected with 0 10 mg of reserpine per kg of b w i m every 24 hrs in per cent First injection g ven at 0 The preparations are identical to those in Fig 1 and the diagrams are similarly arranged *The 100 per cent level for each series is the mean secretory activity* in histamine units (HU) per mg preparation f 2 control sets of ½ half antra (boxed data

justified to use untreated cats as controls The mean control values in Series I to VI are given *in Fig 1 to 4 (boxed data*

To determine the random variation in the assay technique each set of preparation in Series II was assayed twice on each of 4 cats The standard deviation was calculated by analysis of variance Snedecor 1956 and amounted to $\pm$ 5 6 HU per mg of preparation Calculated on the values for antral gastrin activity the standard deviation amounted to $\pm$ 400 HU per g of mucosa

The random variation in the extraction procedure — performed on the same occasion — and in the assay technique is reflected in the difference in the values for secretory activity as between the paired A and B sets of the preparations In no instance (19 pairs of sets, Series I to VII did analysis of variance reveal any significant ($P > 0\ 05$) difference between the activity of the preparations from the paired A and B sets nor between the activity per g of antral mucosa.

Effect of 0 10 mg of reserpine per kg of b w on antral gastrin The mean yield of gastrin preparation in mg per g of mucosa was not appreciably altered by a single reserpine injection or by repetition of the injection daily for 2 4 or 8 days The yield in per cent of the mean for the controls is shown in Fig 1

The mean secretory activity of preparations from untreated and reserpine injected cats is given in Fig 2 The mean activity of preparations from antra removed *6 hours* after a single i m reserpine injection tended to exceed the activity of the control

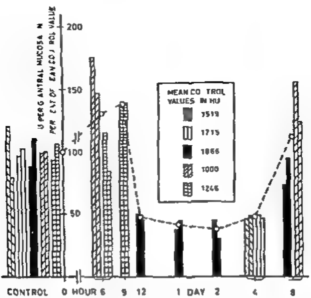

Fig 3 Mean gastrin activity per g antral mucosa (frozen) in nonvagotomized cats injected with 0 10 mg of reserpine per kg of b v i m every 24 hrs in per cent First injection given at 0 The 100 per cent level for each series is the mean gastrin activity in histamine un ■ (HU) per g mucosa (frozen) in 2 control sets of 5 half antra (boxed data) HU per g mucosa is calculated from absolute data in Figs 1 and 2 the diagrams are similarly arranged

preparations in one series the increase amounted to 60 per cent (8 0 HU per mg of preparation) in the other to 13 per cent (2 2 HU per mg) The former increase was significant ($P < 0\ 01$) the latter not ($P > 0\ 05$) The preparations from antra removed 9 hours after reserpine became on the average 34 per cent (6 0 HU per mg) more active than the corresponding control preparations ($P < 0\ 05$ almost significant) but extension of the interval to 12 and 24 hrs led to a reduction in the mean activity by respectively 51 (13 1 HU per mg $P < 0\ 05$) and 60 (15 3 HU per mg $P < 0\ 01$) per cent One reserpine injection daily for 2 or 4 days caused about the same reduction of activity as a single injection in 24 hrs the reduction after 2 injections amounted to 62 per cent (16 0 HU per mg $P < 0\ 01$) and after 4 injections to 65 per cent (16 9 HU per mg $P < 0\ 001$ highly significant) in one series and 52 per cent (12 ? HU per mg $P < 0\ 001$) in the other (white bars) After 8 days in spite of reserpine daily the mean activity of the preparations did not in two series differ significantly ($P > 0\ 05$) from that of the corresponding controls

The antral gastrin activity (HU per g of mucosa) at different time intervals after reserpine and after repeated reserpine injections (Fig 3) ran parallel with the secretory activity of the preparations since the yield of the preparations by weight remained almost unaltered by reserpine Initially a single reserpine injection tended to increase antral gastrin activity after 6 hrs the mean activity was significantly ($P < 0\ 01$) increased by 62 per cent (670 HU per g of mucosa) in one series but unchanged in the other and after 9 hrs the increase amounted to 38 per cent (480 HU per g $P < 0\ 05$) In 12 and 24 hrs reserpine reduced the mean antral gastrin activity by respectively 52 (970 HU

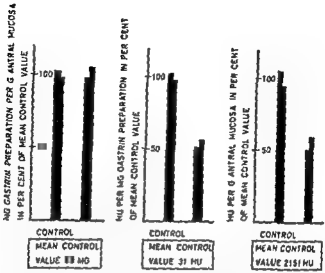

Fig 4 Mean yield of gastrin preparation per g antral mucosa (frozen) of nonvagotomized cats 24 hrs after a single i m injection of 2 0 mg of reserpine per kg of b w in per cent (left diagram) m an secretory activity per mg preparation in per cent (middle diagram) and mean gastrin activity per g antral mucosa in per cent (right diagram) Each bar represents the mean value for 5 half antra of 5 cats (see Methods) The preparations were made simultaneously (Series VI) The 100 per cent level in each diagram is the mean value for 2 control sets of 5 half antra (boxed data) expressed in mg preparation per g antral mucosa (left) in histamine units (HU) mg preparation (middle) and in HU per g antral mucosa (right)

per g $P < 0.05$) and 59 (1100 HU per g $P < 0.01$) per cent Reserpine injected daily for 2 or 4 days produced approximately the same reduction of mean activity as did a single injection in 24 hrs Reserpine for 2 days reduced activity by 63 per cent (1170 HU per g $P < 0.01$) and reserpine for 4 days by 53 per cent (800 HU per g $P < 0.01$) in one series and 52 per cent (890 HU per g $P < 0.001$) in the other (white bars) After [illegible] days the mean antral gastrin activity had returned to normal in two series in spite of reserpine daily (no significant difference from the controls $P > 0.05$)

In several species investigated including the cat no gastrin activity has been detected in the corpus mucosa of the stomach (Komarov 1938 1942 Uvnäs 1942 1943 1945 Harper 1946 Gregory and Tracy 1961) by the different gastrin extraction procedures employed The possibility was therefore considered — although it did not seem very likely — that the low antral gastrin activity after reserpine was due to the mucosa specimens happening to contain some corpus mucosa The proximal borders of specimens from the cats injected with reserpine for 2 days were therefore examined histologically They showed the same appearance as the antral mucosa just proximal to the pyloric sphincter

Effect of 2 0 mg of reserpine per kg of b w on antral gastrin A single i m injection of reserpine (Fig 4) produced in 24 hrs no change in the mean yield of gastrin prepara tions [illegible] mg per g of mucosa The mean secretory activity of the preparations was reduced by 46 per cent (14 3 HU per mg) and the mean antral gastrin activity by 45 per cent (970 HU per g) These reductions were significant (P [illegible] 0 01)

Effect of 0 03 mg of reserpine per kg of b w on antral gastrin The mean yield of gastrin preparations from antra removed 24 hours after i v reserpine was 21 per cent less than the mean yield from the control antra (74 mg per g but neither the secretory activity

TABLE I Secretory activity in histamine units (HU) per mg of preparation of gastrin preparations from the A and B halves of the duodenal mucosae of nonvagotomized cats untreated (control) and treated with reserpine for 4 days (0 10 mg per kg of b w daily i m) and the estimated duodenal gastrin activity in HU per g frozen mucosa

Preparations assayed on cat no	Secretory activity in HU per mg preparation				Secretory activity in HU per g duodenal mucosa (frozen)			
	Control		After reserpine treatment		Control		After reserpine treatment	
	Set A	Set B	Set A	Set B	Set A	Set B	Set A	Set B
137	1.5	1.5	0.7	<1.0	109	103	65	< [illegible]
147	1.3	2.7	<0.7	<0.7	94	186	< 65	< 62
145	2.0	3.3	1.5	1.7	145	297	140	149
Mean	1.6	2.5	<1.0	<1.1	116	172	< 90	<100
Total mean	2.1		<1.1		144		<95	

[1] Explained in methods

[2] The mucosa of proximal duodenum from 3 cats

of the preparations (21 6 HU per mg) nor the antral gastrin activity (1260 HU per g) differed significantly ($P > 0.05$) from the corresponding controls (17 1 HU per mg 1270 HU per g)

Gastrin Preparations from the Duodenal Mucosa of Nonvagotomized Cats

The mean yield of the preparations from the control sets of duodenal mucosa was 71 mg per g of mucosa The secretory activity of the control preparations and the duodenal gastrin activity of untreated cats (Table I) was approximately 10 times less than the activity of corresponding preparations from antral mucosa and antral gastrin activity (Series 1)

Effect of 0 10 mg of reserpine per kg of b w on duodenal gastrin After reserpine daily i m for 4 days the mean yield of preparations exceeded that of the controls by 27 per cent or 19 mg per g of mucosa The mean secretory activity was less after reserpine treatment than the activity of the controls both when expressed in HU per mg of preparation and in HU per g of duodenal mucosa (Table I) Due to the low secretory activity of the preparations from reserpine treated cats the figures for activity represent only rough estimates and have not been statistically analyzed

Gastrin Preparations from the Antral Mucosa of Vagotomized Cats

The yield of preparation was not appreciably influenced by vagotomy neither the secretory activity of the preparations nor the antral gastrin activity was significantly ($P > 0.05$) altered (Fig 5)

Effect of 0 10 mg of reserpine per kg of b w on antral gastrin After reserpine i m daily for 2 days (Fig 5) the yield of preparation from nonvagotomized cats was 5 per cent higher and from vagotomized cats 11 per cent less than from untreated nonvagotomized and vagotomized cats respectively The secretory activity of the preparation from

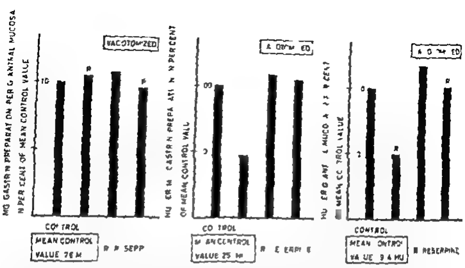

Fig 5 Mean yield of gastrin preparation per g antral mucosa (frozen) of nonvagotomized and vagotomized cats 24 hrs after a period of 2 days with one reserpine injection per day (0 10 mg per kg of b w i. m) in per cent (left diagram) mean secretory activity per mg preparation in per cent (middle diagram) and mean gastrin activity per g antral mucosa in per cent (right diagram) Each bar represents the mean value for 3 antra The preparations were made simultaneously (Series VIII) The 100 per cent level in each diagram is the mean value for the control set of 3 antra from the nonvagotomized cats (boxed data) See text to Fig 4

nonvagotomized cats treated with reserpine was 53 per cent (13 4 HU per mg $P <$ 0 001) less than that from untreated cats whereas in the same series reserpine treatment of vagotomized cats did not significantly ($P > 0\ 05$) alter the activity the mean activity of the preparation from untreated vagotomized cats amounted to 27 4 HU per mg and from reserpine treated vagotomized cats to 26 5 HU per mg Similarly reserpine treatment highly significantly ($P < 0\ 001$) reduced the antral gastrin activity of nonvagotomized cats by 50 per cent (970 HU per g) but produced no significant ($P > 0\ 05$) reduction of the antral gastrin activity of vagotomized cats the small and insignificant reduction being due mainly to the different yield of preparation

Gastrin Preparations from the Duodenal Mucosa of Vagotomized Cats

The yield of preparation from nonvagotomized cats was 93 mg per g of duodenal mucosa and from vagotomized cats 85 mg per g The figures for the secretory activity of the preparation from both nonvagotomized and vagotomized control cats and the corresponding figures for duodenal gastrin activity agreed well with those from the controls given in Table I

Effect of 0 10 mg of reserpine per kg of b w on duodenal gastrin After reserpine treatment for 2 days the yield of preparation from the nonvagotomized and vagotomized cats was respectively 82 and 81 mg per g of duodenal mucosa Reserpine treatment of the nonvagotomized cats reduced the secretory activity of the preparation and the duodenal gastrin activity to about the same extent as did 4 days of treatment (cf Table I) No reduction of either activity was produced by the reserpine treatment of vagotomized cats

In 50 nonvagotomized cats subjected to single or repeated reserpine injections (0 10 mg per kg of b w Series I to V) one or several macroscopic ulcers were observed in the antral specimens of 23 cats (46 per cent) and in the proximal 4 cm of the duodenum of 9 cats (18 per cent) Ulcers were never observed in the proximal half of the stomach None of the 10 animals injected daily with reserpine for 8 days had macroscopic ulcers in the stomach or the duodenum

Discussion

The present investigation demonstrated that an i m injection of 0 10 mg of reserpine per kg of b w after a delay of more than 9 hrs reduced the antral gastrin activity in cats After 24 hrs the reduction was significant amounting to about 60 per cent no further reduction was produced by repeating the injection every 24 hrs for 2 or 4 days After 8 days antral gastrin activity had returned to normal in spite of reserpine daily An i m injection of 2 0 mg of reserpine per kg reduced the activity in 24 hrs about as much as 0 10 mg per kg suggesting that the maximum reduction attainable by reserpine was approximately 60 per cent 0 03 mg per kg infused i v did not significantly alter activity from normal Antral gastrin activity was unaltered by vagotomy and in vagotomized cats 0 10 mg of reserpine per kg i m daily for 2 days produced no significant reduction of activity

The yield of gastrin preparation in mg per g of antral or duodenal mucosa was fairly uniform and uninfluenced by reserpine the changes in the secretory activity of the preparations and in antral (duodenal) gastrin activity accordingly run almost parallel The unaltered yield in mg was only to be expected since the pure gastrin by weight probably comprises only a very small fraction of the preparation (cf Gregory and Tracy 1964)

The secretory activity of corresponding A and B sets from the antral mucosae did not differ significantly whether activity was expressed in HU per mg of preparation or in HU per g of mucosa This demonstrated that the method of gastrin extraction (when the extracts were prepared simultaneously) and the assay technique used were reliable and it was thus possible to use undivided antra in the series with vagotomized cats

Earlier reports (Komarov 1938 1942 Uvnas 1945 Harper 1946) of a gastrin like HCl-stimulating factor in the duodenal mucosa were confirmed also for cats The mean duodenal gastrin activity of the 4 centimetres of duodenum analyzed amounted in the present study to roughly one tenth the antral gastrin activity but the distal distribution of the gastrin activity within this duodenal segment was not investigated Reserpine (0 10 mg per kg i m) daily for 2 or 4 days reduced the duodenal gastrin activity but no estimate was made of the magnitude of this reduction owing to the low secretory activity of the preparations Vagotomy did not alter the duodenal gastrin activity and reserpine for 2 days produced no reduction of activity in vagotomized cats

There are no previous reports of experimentally induced measurable changes in antral and duodenal gastrin activity Lythgoe Dickinson and Waddell (1961) obtained no reduction of the secretory activity of preparations from the antral mucosa of dogs 24 hrs after reserpine (5 mg per kg of b w) Apart from the possibility of a species difference these authors administered reserpine by the mouth and their methods for the extraction of gastrin and for the assay of the secretory activity of the extracts differed from ours

Reserpine produced a reduction of antral and duodenal gastrin activity only in cats with intact vagal nerves Since evidence has recently been presented for a vagal action of reserpine on the stomach causing release of gastric histamine in rats (Kim and Shore 1963) and an increased responsiveness of the parietal cells to exogenous histamine and

gastrin in cats (Emås 1963 1964) the reduction of activity probably means that reserpine causes gastrin release from the antral and duodenal mucosa by central vagal activation However a decrease in gastrin synthesis or gastrin storage capacity might contribute to or be responsible for the reduction The present findings provide further evidence to those based on gastric secretory studies (Uvnäs 1942 Maung Pe Thein and Schofield 1959) for vagal release of gastrin and they support the previous suggestion that reserpine treatment interferes with the gastrin mechanism (Emås 1963) The maximum reduction of antral gastrin activity was about 60 per cent The residual gastrin activity suggests continuing gastrin synthesis submaximal vagal activation and/or a pool of gastrin resistent to vagal impulses Whether the unaltered or possibly even slightly increased antral gastrin activity within 9 hrs after reserpine, i e before the reduction of activity occurred is due to gastrin being both released and synthetized or to gastrin not being released is a question that cannot yet be answered The antral gastrin activity returned to normal after 8 days despite reserpine treatment This normalization of activity — confirmed in a second series of experiments — was unexpected, since the increased vagal tone of reserpine treated cats as estimated by the elevation of secretory responses to exogenous histamine and gastrin preparations (Emås 1963 1964) remained when treatment was prolonged beyond 8 days (Emås 1963) The returning gastrin activity could represent an adaptation of the mechanisms involved in gastrin release and/or in gastrin synthesis or storage to reserpine treatment or to the enhanced vagal tone The significance of the vagal tone for the antral and duodenal gastrin activity is unknown but the present study demonstrates that the values for gastrin activity were normal 1 to 2 months after vagotomy

It has been suggested previously that a cholinergic drug (methacholine) and vagal (insulin hypoglycemia) might release lesser amounts of gastrin in cats treated with reserpine for 3 days or more than in untreated cats (Emås 1963) However a small supply of gastrin in the antral mucosa of reserpine treated cats does not appear to be the explanation for this since the antral gastrin activity after 8 days of treatment did not differ significantly from that of untreated cats

In nonanesthetized gastric fistula cats a continuous i v infusion of 1 to 3 μg of histamine dihydrochloride per minute equivalent in stimulating acid secretion to 1 to 3 HU per minute maintained a stable HCl secretion for hours (Emås 1962) A release of gastrin corresponding to the mean 12 hour reduction of antral gastrin activity following 0 10 mg of reserpine per kg (970 HU per g of mucosa) is accordingly sufficient to maintain a continuous gastric hypersecretion of acid for more than 12 hours supposing the potency of endogenous gastrin to remain essentially unaltered by passage through the liver as that of impure exogenous gastrin is in dogs (Gillespie and Grossman 1962) An i v 15 minute infusion of 0 01 to 0 03 mg of reserpine per kg elicits in non anesthetized cats a gastric acid secretory response lasting for more than 12 hours (Emås to be published) although there was no measurable reduction of antral gastrin activity 24 hrs after 0 03 mg of reserpine per kg i v in cats with intact vagal nerves This did not exclude that the small i v dose of reserpine causes gastrin release but it could suggest that reserpine stimulates acid secretion also by other mechanisms than by vagal release of gastrin The intra antral pH was presumably less in the gastrin studies than in the gastric secretory studies since continuous drainage of the stomach via a gastric cannula was applied only in the latter In the gastrin studies a low intra antral pH could therefore have suppressed the vagal release of gastrin (for references see Andersson and Olbe 1964) induced by i v reserpine Nevertheless an unpuls-

lished experiments 0.03 mg of reserpine per kg i v produced a long lasting hyper secretion of acid in nonanesthetized cats also when the gastric cannula was closed during the first 3 hours after reserpine

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From the Department of Pathology Section II University of Helsinki Finland

Tissue Eosinophils of Rat under the Influence of Cysteine, Lysine and X-Irradiation[1]

By

LARS R HOLSTI and TAPIO RYTOMAA

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Abstract

Holsti L R and T Rytomaa *Tissue eosinophils of rat under the influence of cysteine lysine and X irradiation* Acta physiol scand 1965 63 370—376 — Cysteine and lysine were given to rats by stomach tube either alone or before whole body irradiation and the tissue eosinophils were ... Repeated daily administration of cysteine and lysine for a week caused a significant ...rease in the eosinophil count of the skin duodenum and colon Short term amino acid treatment caused no changes in the tissue eosinophil count Whole body irradiation alone and after amino acid pre treatment also caused a significant decline in the tissue eosinophil count This concurs with observations that long term treatment with ACTH and cortisone lowers the number of tissue eosinophils (Wegelius and Teir 1958 Rasanen 1961) The principal extra medullary amount of eosinophils of the organism is in the tissues The significance of these cells for the organism is that they constitute one of its defensive and protective mechanisms (Rytomaa 1960) There seems to be a relationship between the number of tissue eosinophils and the radio-protective effect of cysteine and lysine established earlier (Holsti 1960)

Eosinophil granulocytes are formed in the bone marrow The principal amount of extramedullary eosinophils of the organism is in the tissues and circulating eosinophils constitute a very small part of these (Rytomaa 1960) Blood eosinophilia is obviously just a phase of transport from the bone marrow to consumption in the tissue (Rytomaa 1960)

On the basis of the available data (Foot 1963) the production of eosinophils in rat can be calculated to about 1 5 10 cells per 100 g b w in 24 hrs and in the state of equilibrium this must equal elimination It has been assumed that the gastrointestinal tract is the main place of elimination of granulocytes and available information actually favors this theory both in respect to neutrophils and eosinophils (Rytomaa 1960 Teir et al 1963)

[1] This study was supported by the Sigrid Juselius Foundation Helsinki and the Damon Runyon Memorial Fund (DRG-644) New York

Single whole body irradiation causes eosinopenia in the blood (Beaumariage 1955 Soveny et al 1961) The number of eosinophils in the blood decreases sharply when an excess of glucocorticoids is secreted by the suprarenal cortex, and this happens also in connection with irradiation (Bacq and Alexander 1961) Glucocorticoids cause transient eosinophilia in the tissues at the same time as eosinopenia is present in the blood (Gross 1957 Wegelius and Teir 1958 Sundell 1960) However, no simple relationship exists between these two phenomena (Rytomaa 1960)

Some experimental results suggest that the blood eosinopenia induced by glucocorticoids could be partly due to various stress metabolites including amino acids (Aschkenasy 1959) Intraperitoneally administered amino acids also cause blood eosinopenia (Aschkenasy 1959)

It has been shown that the amino acids cysteine and lysine protect animals against the lethal effect of whole body irradiation (Holsti 1960) The aim of the present paper was to investigate the effects of cysteine and lysine alone and prior to irradiation as well as the effect of irradiation alone on the eosinophil count in the skin duodenum and colon In view of all the effects of irradiation and amino acids on blood eosinophils and the apparent defensive mechanism of the eosinophil cells a study on these lines seemed to be of certain interest

Material and methods

234 female rats of the Sprague Dawley strain age 5 months and weight 160—240 g were used. They were caged at the Department for 10—14 days before the experiments They were fed ad libitum a mixture described in detail elsewhere (Holsti 1960) L-cystein hydrochloride and L (+) lysine dihydrochloride (Hoffman La Roche) were employed in the experiments The amino acids were given in water solution by stomach tube into an empty stomach always at the same time of the day The cysteine solution contained 150 mg as cysteine per ml (15 per cent weight per volume) the lysine solutions were equimolar to the cysteine solutions Both solutions were adjusted to pH 2 only fresh solutions were employed In the irradiation experiments, the amino acid solutions were administered 45 min before irradiation

Irradiation was given at 190 kV and 16 mA HVL = 1 60 mm Cu The measured dose was 400 r The animals were exposed to whole body irradiation for 22 min in groups of 5—8 each rat in its own well ventilated cardboard box

Tissue specimens were removed immediately after decapitation and fixed in Bouin s fluid The paraffin treated specimens were sectioned at 5 μ the duodenum and colon crosswise. The preparations were then stained with hematoxylin-eosin Using a magnification of 12 1 1/2 × 45 the eosinophils in 50 visual fields were counted from the dermis of the skin the lamina propria of the duodenum and colon

The Kruskall Wallis test and Wilcoxon s test were employed for the statistical treatment of the results

Results

Effect of Amino Acids only

Successive doses of cysteine and lysine were given to 72 rats In the first experiment the dosage was 675 mg of cysteine per kg b w 3 times daily at 1 hr intervals with the lysine solution equimolar to the cysteine solution each dose 750 mg per kg b w In the second experiment the dosages were 1 250 mg/kg b w cysteine (maximal tolerated dose and the equimolar amount of lysine was 1 500 mg/kg b w given daily into an empty stomach for a week

The administration of 3 successive doses with 1 hr intervals did not cause any significant variations in the number of eosinophils in the dermis or lamina propria of the duodenum and colon (Table I)

TABLE I Tissue eosinophils after 3 successive doses of cysteine and lysine

Treatment group	No of rats	Mean eosinophils per 50 visual fields								
		Dermis			Duodenum			Colon		
		Days after WBI								
		1	3	8	1	3	8	1	3	8
		Day								
Cysteine 3	15	19	29	17	342	387	315	219	297	203
Lysine × 3	15	17	39	34	649	342	407	291	249	232
Control saline	15	9	16	28	491	531	469	285	214	259
Significance of differences between groups										

TABLE II Tissue eosinophils after long term treatment with cysteine and lysine

Treatment groups	No of rats in group	Mean eosinophils per 50 visual fields								
		Dermis			Duodenum			Colon		
		1	3	8	1	3	8	1	3	8
		Day								
Cysteine 7	21	20	28	22	184	272	276	266	308	444
Lysine 7	21	16	26	19	240	317	452	355	482	389
Untreated controls	21	33	38	45	351	302	294	464	433	446
Significance of differences between groups										

= P 0.05
P 0.01

Repeated daily administrations of cysteine and lysine for a week caused a decrease in the eosinophil count on the first day in dermis (P 0.05) the lamina propria of the duodenum (P 0.05) and colon (P 0.01) as compared with the control animals (Table II) The eosinophil count rose to nearly the normal level on the third day and to the normal level on the 8th day A pronounced over-shooting of the control level occurred on the 8th day in the animals treated with lysine

TABLE III Tissue eosinophils in rats irradiated only and pre treated with protective doses of cysteine and lysine prior to irradiation

Treatment group	No of rats	Mean eosinophils per 50 visual fields								
		Dermis			Duodenum			Colon		
		Days after irradiation								
		1	3	8	1	3	8	1	3	8
		Days								
Cysteine × 3 + WBI	22	10	11	2	182	96	28	253	80	27
Lysine × 3 + WBI	24	31	14	1	154	190	23	308	103	17
WBI 700 r alone	40	22	14	5	223	174	80	[illegible]63	1[illegible]5	34
Untreated controls	25	33	38	45	351	302	294	464	433	446
Significance of differences between treated and untreated groups										
Significance of differences between treated groups										

P 0 05
P 0 01
P 0 001

Effect of Whole Body Irradiation alone and in combination with Amino Acids

Forty rats were irradiated with 700 r Seven rats were decapitated 1 3 and 8 days after the exposure Twentytwo rats were given cysteine and 24 rats lysine in 3 successive doses prior to irradiation. This amino acid dosage was known to be radioprotective Holsti 1960) Twentyfive additional rats were given cysteine and 25 rats lysine in daily doses for 1 week prior to irradiation This dosage had no protective effect (Holsti 1960) The irradiation was given 45 min after the last amino acid dose

Both irradiation alone and irradiation after amino acid pre treatment caused a statistically significant decrease in the eosinophil count on the first day after irradiation in the lamina propria of the duodenum P < 0 01 and the colon P 0 001) Table IV) On the third day the number of eosinophils was significantly lower in all the organs investigated and also on the eight day Table III and IV With 3 exceptions there was no statistically significant difference between the groups given irradiation alone and the groups pretreated with amino acids Longterm treatment with cysteine and lysine for 1 week before irradiation produced a lower eosinophil count in the lamina propria of the colon and the duodenum as compared with rats given irradiation only However only two of the differences as indicated in Table IV were statistically significant at a level of P 0 05 A similar difference was noted in the duodenum of rats treated 3 times with amino acids before irradiation Table III

TABLE IV Tissue eosinophils in rats irradiated only and given long term pre treatment with a non protective dosage of cysteine and lysine

Treatment group	No of rats in group	Mean eosinophils per 50 visual fields								
		Dermis			Duodenum			Colon		
		1	3	[illegible]	1	3	8	1	3	8
		Day								
Cysteine × 7 + WBI	25	30	10	7	125	109	36	149	[illegible]	57
Lysine × 7 + WBI	25	13	6	5	118	138	34	191	79	57
WBI 700 r alone	40	22	14	5	223	174	[illegible]	263	175	34
Untreated controls	21	33	38	45	351	302	294	464	433	44[illegible]
Significance of differences between treated and untreated groups			a	1						1
Significance of differences between treated groups							1			

1 = P < 0 05
2 = P < 0 01
3 - P 0 001

Discussion

Working from the suggestion that the blood eosinopenia induced by glucocorticoids could be due partly to various metabolites including amino acids discharged into the circulation following the tissular action of the hormones Aschkenasy (1959) showed that almost all the amino acids existing in serum proteins e g cysteine and lysine induced blood eosinopenia in intact rat when injected i p In the present experiments long term peroral treatment with both cysteine and lysine caused a statistically significant decrease in the number of tissue eosinophils in the dermis and in the lamina propria of the duodenum and colon as compared with the controls Long term treatment with ACTH and cortisone also causes a decrease in tissue eosinophils in rat gastrointestinal tract (Wegelius and Teir 1958 Sundell 1960 Rasanen 1960 as well as in all other organs studied (Gross 1954) Short term treatment with cysteine and lysine (3 successive days) produced no detectable differences in the number of tissue eosinophils although amino acids have a weaker eosinopenic effect in adrenalectomised rats than in intact rats (Aschkenasy 1959) Furthermore some observations suggest that cysteine may have a stimulating effect on the adrenal cortex (Hoffmann *et al* 1955)

Whole body irradiation with 700 r caused a significant fall in the tissue eosinophil count of dermis and the lamina propria of the duodenum and colon Irradiation causes

overactivity of the suprarenal cortex and the presence of an excess of glucocorticoids in the blood a few hours after irradiation may accentuate the fall in the number of circulating eosinophils (Beaumariage 1955 Soveny1 *et al* 1961) Administration of a single dose of cortisone provokes an increase in the tissue eosinophil count of rat and guinea pig gastrointestinal tract within 4 hours (Gross 1957 Sundell 1960 Wegelius and Teir 1958) Tissue eosinophilia appears at the same time as blood eosinopenia under the influence of glucocorticoids The latter phenomenon does not however directly cause the transient tissue eosinophilia as clearly indicated by the changes in the total cell numbers in the blood and tissues respectively (Rytomaa 1960) The behaviour of tissue eosinophils after irradiation is currently under study at this laboratory (Cederberg)

Blood eosinophils decrease in number after a single whole body irradiation both in rat (Beaumariage 1955 Soveny1 *et al* 1961) and in man (Lasser and Stenstrom 1954a) The same phenomenon has also been established after fractionated WBI in rat [5 fractions in 14 days] (Widman 1958) However the blood eosinophil count of patients with carcinoma rises during local fractionated radiotherapy even above normal (Lasser and Stenstrom 1954 Morczek and Christoph 1958)

Several observations show liberation of histamine (Bacq and Alexander 1961 Beaumariage 1955 Veninga and De Boer 1963) and an elevated blood histamine level after irradiation (Bacq and Alexander 1961 Lasser and Stenstrom 1954 b) An injection of Histamine produces a decrease in the number of blood eosinophils in rat (Halpern and Benos 1951) and eosinophilia in the small intestine (Sundell 1958) In addition to histamine 5 hydroxytryptamine is also liberated on exposure to ionizing radiation (Bacq and Alexander 1961 Veninga and Brinkman 1962) Observations made by Rose *et al* 1958 and Zaratzian 1959 indicate that liberated endogenous histamine and 5-hydroxytryptamine may play an important role in corticoid eosinopenia The same mechanism may also have importance in irradiation-eosinopenia by increasing their need and consequent consumption as compared with normal condition

In the present experiments there were detectable differences between the eosinophil counts in the colon and the doudenum of animals pre treated with cysteine and lysine before irradiation and those given irradiation alone It is noteworthy that tissue eosinophil count remains very low for at least 8 days after irradiation in the organs examined while the mitotic indices return to normal or above on the third day in the duodenum and on the eight day in the skin (Holsti 1960) This is probably partially due to the continuous high consumption of eosinophils after irradiation and obviously to the inability of eosinopoiesis to get rapidly under way again Bone marrow is restored in rats after a high dose of irradiation only after 3 weeks (Fliedner and Stodtmeister 1962)

Most of the radioprotective agents e g cysteine protect the hematopoietic system and stimulate the regeneration of hematopoietic tissue (Holsti 1960 Thomson 1962) It has been demonstrated previously that 3 successive doses of both cysteine and lysine protect rats against radiation induced death whereas pre treatment for a week has no protective effect (Holsti 1960) In the present experiment the tissue eosinophil count showed no detectable change after 3 successive doses of cyteine and lysine but after a week s pre-treatment the count was lower in all the organs studied Hence there seems to be some kind of relationship of eosinophils in tissues and the radioprotective effect of cysteine and lysine This is obviously consistent with the view that eosinophil cells have a specific defensive function in the organism against external noxae

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From the Institute of Biological Chemistry University of Copenhagen Denmark

The Effects of Surfactants on the Potential, Short-circuit Current, and Ion Fluxes Across the Isolated Frog Skin

By

George D Webb

Received 7 July 1964

Abstract

Webb G D *The effects of surfactants on the potential short-circuit current and ion fluxes across the isolated frog skin* Acta physiol. scand 1965 *63* 377—384 — It has been shown previously that ionic surface active agents (surfactants) depolarize nerve and muscle membranes and the isolated frog skin The isolated frog skin was used in the present studies. Measurements of skin potential, short circuit current and of Na K and Cl fluxes were made to determine the relative roles of inhibition of active Na transport and increases in passive ion permeability in producing the depolarization. The electrical data were in agreement with the flux data in demonstrating that lower concentrations of some surfactants (0.5 mM) generally inhibit the active Na transport process but have little effect on passive ion permeability At higher concentrations (2 mM or more) surfactants produce an increase in permeability in addition to inhibiting the Na pump The outside surface of the frog skin was more sensitive to the surfactants than was the inside

It is well established that ionic surface active agents (surfactants depolarize nerve and muscle cell membranes (Adelman and Kishimoto 1961 Hober 1947 Wasano Ogata and Goto 1956) It has also been demonstrated that surfactants depolarize isolated frog skin (Schoffeniels Gilles and Dandrifosse 1962) The depolarization might be due either to an increased passive permeability to ions or to an inhibition of the active sodium transport mechanism or both The present study was an attempt to determine the roles played by these two possible actions of surfactants

The inward facing membranes of the epithelial cells of frog skin are very similar to nerve and muscle membranes in being permeable (when at rest) to K and Cl but relatively impermeable to Na and in being equipped with a metabolically driven "pump" which transports Na out of the cells (Koefoed Johnsen and Ussing 1958 Ussing 1960)

The isolated frog skin offered several advantages for the present experiments 1) a large backlog of data has been accumulated for this preparation 2) the large surface

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It is well established that ionic surface active agents (surfactants) depolarize nerve and muscle cell membranes (Adelman and Kishimoto 1961, Hober 1947, Wasino, Ogata and Goto 1956). It has also been demonstrated that surfactants depolarize isolated frog skin (Schoffeniels, Gilles and Dandrifosse 1962). The depolarization might be due either to an increased passive permeability to ions, or to an inhibition of the active sodium transport mechanism, or both. The present study was an attempt to determine the roles played by these two possible actions of surfactants.

The inward facing membranes of the epithelial cells of frog skin are very similar to nerve and muscle membranes in being permeable (when at rest) to K and Cl but relatively impermeable to Na and in being equipped with a metabolically driven "pump" which transports Na out of the cells (Koefoed-Johnsen and Ussing 1958, Ussing 1960).

The isolated frog skin offered several advantages for the present experiments: 1) a large backlog of data has been accumulated for this preparation; 2) the large surface

area available facilitates ion flux measurements and 3) the spontaneous electric potential and the short-circuit current across the isolated frog skin are easily measured because no microelectrodes are required.

Methods

All experiments were done on the abdominal skin of *Rana temporaria*. The frogs were pithed and the skin was removed, washed in Ringer, and mounted as a flat sheet between lucite chambers. 7.06 cm^2 of skin were exposed to the 25 ml of Ringer which circulated on each side.

A Ringer solution of the following composition was used: 115 mM Na, 2.5 mM K, 1.0 mM Ca, 117 mM Cl and 2.5 mM HCO_3. When equilibrated with room air this solution had a pH of 8.3. The addition of surfactants to the Ringer did not significantly affect the pH.

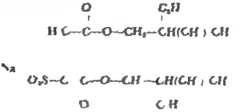
The following surfactants were used:

Anionic

sodium lauryl sulfate (SLS)

$CH_3(CH_2)_{11}$—O—SO_3Na

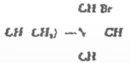
Aerosol OT (AOT) = dioctyl sodium sulfosuccinate



Cationic

cetyl pyridinium chloride (CPC)

cetyltrimethylammonium bromide (CTAB)



The conical chambers used and the correction necessary because the potential bridges are located 5 mm away from the skin have been described by Koefoed-Johnsen, Ussing and Zerahn (1952). The other apparatus used and the method for short-circuiting the skin have been described in detail by Ussing and Zerahn (1951). In the experiments in which Na or Cl fluxes were measured, the skins were kept continuously on short-circuit (zero potential) except that every ten minutes the skins were put on open-circuit for seven seconds, at the end of which the skin potential was measured. In all other experiments the skins were kept on open-circuit except that every ten minutes the skins were short-circuited for 4.5 seconds, at the end of which the short-circuit current was determined. Potential readings were not taken during open-circuit experiments until at least five minutes after shorting the skin, because of the temporary increase in potential that usually occurs after short-circuiting the skin, as has been described by Teorell and Schöffeniels (19?).

After being mounted between the chambers the skins were allowed to equilibrate with Ringer for two hours before the regular ten minute readings were begun. A control period of 40 min or longer followed the two hour equilibration period. Short-circuit current and potential were usually quite steady during the control period. Surfactant was then added. Solutions were changed four times preceding a recovery period to assure removal of surfactants.

Na influx (flux from the skin into the inside solution) and efflux (flux from the skin into the outside solution) were measured simultaneously using ^{24}Na and ^{22}Na as described by Koefoed

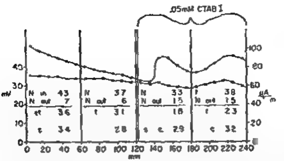


Fig 1 Changes in potential short-circuit current and Na fluxes produced by exposure of the inside surface of a frog skin to 0.5 mM CTAB In this figure and in Fig 2 the vertical lines mark the time of collection of samples for counting except the line at 12? min which is the time at which CTAB was added to the inside solution in = influx (given in µ mol/hr) out = efflux and s c c = short-circuit current given as equivalent µ mol Na/hr The skin was held on short circuit current during this experiment and the one illustrated in Fig 2 In this and in all succeeding figures each ■ represents a measurement of skin potential in mV and each ● represents a measurement of short circuit current in µ A/7 06 cm

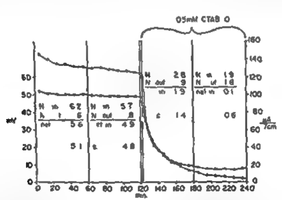


Fig 2 Changes in potential (■) short-circuit current (●) and Na fluxes produced by exposure of the outside surface of a frog skin to 0.5 mM CTAB

Johnsen Ussing and Zerahn (1952) Cl influx was measured using Cl The use of Cl influx rather than efflux avoided the possible complication of secretion of Cl by the mucous glands (Koefoed Johnsen Ussing and Zerahn 1952) 500 µl radioactive samples were removed every hour and evaporated on aluminum planchets containing a circle of thin lens tissue and 2 mg of glucose A thin window halogen quenched Geiger Muller tube was used for counting Samples were counted for a sufficient length of time so that the standard deviation due to statistical fluctuation of the counts was generally less than 8 of the calculated flux values (the standard deviation of a net count resulting from the subtraction of count N from N is $\sqrt{N + N}$)

K flux was measured using K free Ringer (the 2.5 mM K was replaced with Na) for the outside solution It has been shown in this laboratory that the absence of K in the outside solution does not affect short-circuit current or active sodium transport (Koefoed Johnsen unpublished) Two ml samples were taken every hour and analyzed for K with a Zeiss flame photometer In experiments where surfactant was added to the outside solution samples were

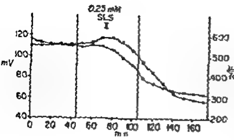


Fig 3 Changes in potential (■) and short circuit current (●) produced by exposing the inside surface of a frog skin to 0.25 mM SLS for one hour. In this and in all succeeding figures the first vertical line marks the time at which surfactant was added to the solution bathing one side of the skin. The second line marks the time at which both inside and outside solutions were changed four times with fresh Ringer solution. In this and the following figures the skins were kept on open circuit during the experiment.

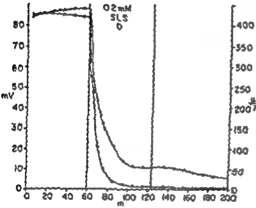


Fig 4 Changes in potential (■) and short circuit current (●) produced by exposing the outside surface of a frog skin to 0.2 mM SLS for 63 min.

taken immediately before and after the surfactant was added. It was found that the surfactant did not influence the flame analysis of K.

A total of 94 experiments were performed. K efflux was measured in 25 of these experiments. Na efflux and influx were measured simultaneously in 15 experiments and Cl influx was measured in [illegible] experiments. Although control periods preceded all experiments four control experiments were done while measuring K efflux and one while measuring Na fluxes to demonstrate that the method of changing solutions or adding surfactant did not affect the measurement.

Results

At higher concentrations ionic surfactants decreased both the skin potential and the short-circuit current. At lower concentrations the effect on the skin potential was often proportionately less than the reduction of the short-circuit current. Some surfactants when applied in low concentrations to the inside of the skin produced an increase in the short-circuit current (Fig 1). This prolonged increase in current was never observed when the surfactants were added to the outside solution (e.g. Fig 2). Fig 3 and 4 also illustrate the markedly different effects observed depending on whether a surfactant was added to the inside or the outside solution. In general the skin potential and the short-circuit current were less affected by surfactant in the inside solution than by surfactant in the outside solution. The action of anionic surfactants was often reversible when the period of application was short (Fig 5 and 6). This reversibility was never

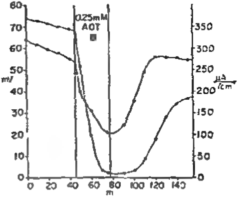


Fig 5 Changes in potential (■) and short-circuit current (●) produced by exposing the outside surface of a frog skin to 0 25 mM AOT for 32 min

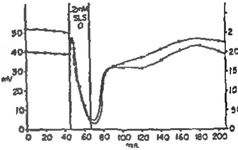


Fig 6 Changes in potential (■) and short-circuit current (●) produced by exposing the outside surface of a frog skin to 0 2 mM SLS for 21 min

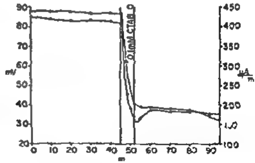


Fig 7 Changes in potential (■) and short-circuit current (●) produced by exposing the outside surface of a frog skin to 0 1 mM CTAB for 7 min

observed with the cationic surfactants tested (e g Fig 7) The temporary increase in skin potential shown in Fig 6 was observed in about 10 % of the experiments Usually it was less marked than in Fig 6 This temporary increase in potential following surfactant treatment has been reported by Schoffeniels *et al* (1962)

Samples of the ion flux data may be seen in Fig 1 and 2 Tables for all the ion flux data are too lengthy to reproduce here In summary it was found that 0 2 mM CTAB

or SLS applied inside or outside always produced a large increase in the efflux of K and Na and the influx of Cl Simultaneously there was always a decrease in net Na influx The application of 0 05 mM SLS or CTAB produced less consistent results K efflux which was normally quite low (generally less than 0 2 μmole/hr) showed no consistent change at all sometimes there appeared to be a decrease Only two Cl influx experiments were performed at this concentration (one with SLS inside and one with CTAB inside) in both of these experiments there was a slight increase in Cl influx Eight Na flux experiments were performed with 0 05 mM SLS or CTAB In all of these experiments there was a slight decrease in net Na influx which was paralleled by a decrease in short-circuit current except in the cases where CTAB was added to the inside solution (e g see Fig 1) In some cases Na efflux increased slightly in others there was no significant change

Discussion

In Fig 1 the short-circuit current increased after the addition of CTAB to the inside solution, whereas the net Na influx decreased A duplicate experiment produced similar results Possibly the current not accounted for by Na transport may have been the result of an outward secretion of Cl by the mucous glands thus CTAB may have had an action similar to that of adrenaline (Koefoed Johnsen *et al* 1952) Many types of ionized amines increase the short-circuit current apparently by increasing the permeability of the outer boundary to Na, thus making more Na available to the pump (Skou and Zerahn 1959) Apparently the CTAB was not acting in this manner or the net Na influx would have increased parallel with the short-circuit current. When CTAB was applied on the outside and when SLS was applied inside or outside the short circuit current (measured in equivalent Na net flux) remained equal to the directly measured net Na influx even when there were large changes in these values This confirms Ussing and Zerahn s observation (1951) that under most conditions the short circuit current is exactly accounted for by the net flux of Na. It is curious that in the South American frog there is sometimes a net inward flux of Cl in a shorted skin so that the short-circuit current is less than the net transport of Na (Zadunausky Candia and Chiarandini 1963)

At higher concentrations 0 2 mM) it is certain that ionic surfactants increased the ion permeability of the frog skin since the measured efflux of K, influx of Cl and efflux of Na all increased without exception upon exposure of the skin to surfactant Also when the isolated frog skin was exposed to high concentrations of surfactants the short-circuit current and the net Na influx were reduced This indicates inhibition of active Na transport. At lower concentrations 0 05 mM the ion fluxes did not show any consistent changes although there was always at least some decrease in the net influx of Na. At the lower concentrations there was always a large decrease in the short-circuit current except when CTAB was applied inside (and once when SLS was applied inside this exception has already been discussed It appears that the principal action of surfactants at lower concentrations was an inhibition of active Na transport assuming that net Na influx and short-circuit current are proportional to active Na transport

Some recent electron microscopic and histochemical data (Farquhar and Palade 1964) and some recent electrophysiological data (Ussing and Windhager 1964 indicate that there are intercellular pathways for the passive diffusion of Na and other ions through the skin If these pathways became "opened" to the outside solution it is

conceivable that short-circuit current and net Na influx could decrease without any actual decrease in active Na transport as Farquhar and Palade s histochemical data indicated that a large part of the active transport of Na may be from the epithelial cells into the intercellular channels which normally are open only to the inside However in the experiments of Ussing and Windhager short-circuit current did not decrease in frog skins in which the "shunt resistance (presumably represented in part by the intercellular channels) had decreased through the action of urea Thus it appears that at least in some circumstances short circuit current is unaffected by changes in "shunt resistance and thus probably remains proportional to active Na transport

Skin potential data combined with the short-circuit current data confirmed that after surfactant treatment short-circuit current probably remained proportional to active transport of Na By dividing the skin potential by the short-circuit current one can obtain a figure for the skin resistance in ohms In the present experiments this resistance usually was within the range of 900 to 2 500 Ω/cm^2 in the untreated skin Probably the skin resistance is roughly inversely proportional to the sum of the permeabilities of the whole skin to all ions Skin resistance showed little change or even increased when the frog skins were exposed to low concentrations of surfactants even though the short-circuit current was often drastically reduced Thus it would appear that active Na transport must have been reduced parallel to the reduction in short circuit current for if the intercellular pathways had become opened to the outside there should have been a decrease in the skin resistance With higher concentrations of surfactant the skin resistance almost always showed a large drop Thus the resistance data are in agreement with the flux data and it seems safe to conclude that lower concentrations of surfactants inhibit the active transport of Na in the frog skin but have little effect on ion permeability At higher concentrations surfactants increase ion permeability in addition to inhibiting active transport It is significant that surfactants inhibited active Na transport in the frog skin before affecting passive ion permeability as many previous investigations on other preparations have emphasized the importance of permeability changes (e g Hodes Palmer and Warren 1960 Jacobj 1970 Nardone *et al* 1956 or Ponder 1947)

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Acta physiol scand 1965 63 385—390

From the Department of Physiological Chemistry University of Lund Sweden

The Metabolism of Fatty Acids in the Rat

III Arachidic Acid

By

GORAN GORANSSON

Received 8 July 1964

Abstract

Goransson G *The metabolism of fatty acids in the rat III Arachidic acid* Acta physiol scand 1965 63 385—390 — 1 C Arachidic acid and 9-10-H palmitic acid in rat serum were simultaneously injected into fasted and refed male rats and the blood and tissue distribution of radioactivity were studied The results suggest that in spite of quantitative differences arachidic acid enters the same principal pathways as palmitic acid The labeled arachidic acid disappeared more slowly from the blood than did the palmitic acid Two minutes after the injection slightly less arachidic than palmitic acid label was recovered from the whole animal In the liver more of the esterified arachidic acid than of the palmitic acid radioactivity was present in triglyceride and less in phospholipid

To establish a reference for a comparative study of the metabolism of different fatty acids palmitic acid dissolved in rat serum was injected i v into rats (Goransson and Olivecrona 1964) Experiments were then performed in which oleic acid and palmitic acid were injected together (Goransson and Olivecrona 1965

The present paper reports the results of experiments in which arachidic acid and palmitic acid where injected simultaneously to rats Arachidic acid is a constituent of most plant and animal lipids but usually occurs in small amounts Cattaneo *et al* 1960 Hatano 1958) Flax seed oil is particularly rich in arachidic acid (Grynberg Szczepanska and Beldowicz 1962) In the rat Goransson and Olivecrona 1964 found that arachidic acid did not exceed 3 of the fatty acids of any of the lipid fractions examined The present work was undertaken to determine whether a fatty acid that is not abundant in the rat (arachidic acid is metabolized in a different way than an abundant one such as palmitic acid which constitutes 10—50 of the total lipid fractions in different organs Goransson and Olivecrona 1964

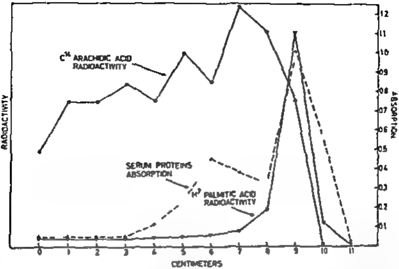

Fig 1 Paper electrophoreses carried out on the injection solution (rat serum) containing C arachidic acid and H palmitic acid Radioactivity was determined by liquid scintillation counting Proteins were determined colorimetrically after staining with amido black

Methods

The nutritional state of the animals the operative procedures and the analytical methods used have been described earlier (Goransson and Olivecrona 1964) The 9 10-H palmitic acid (batch 3 389 mC/mmole) was obtained from The Radiochemical Centre Amersham England It was purified as described earlier (Goransson and Olivecrona 1964) The 1 C arachidic acid (2 0 mC/mmole) was delivered by Calbiochem California USA It was purified by reversed phase chromatography twice using 90 methanol and 10 water as the mobile phase and heptane as the stationary phase The acid was recovered from the acidified methanol water (1 1) solution by extraction with petroleum ether A solution of the two acids to be injected was prepared as described previously (Goransson and Olivecrona 1964 Electrophoresis of the injection solution gave the results shown in Fig 1 where it can be seen that arachidic and palmitic acid have different mobilities The palmitic acid moved mainly with the albumin while the arachidic acid moved largely with globulin In a control experiment with labeled fatty acids not bound to serum protein all of the radioactivity was recovered at the origin

0 5 ml of the freshly made solution was injected into the jugular vein This dose contained approximately 0.2 μeq of labeled arachidic acid and 0 05 μeq of labeled palmitic acid

Results

The percentage of labeled palmitic acid found in the examined lipid fractions agreed with the values found earlier (Göransson and Olivecrona 1964) Accordingly the values reported here are given as C^{14}(arachidic acid)/H^3(palmitic acid) in the sample divided by the same ratio present in the injected material (There is one exception in Fig 2 the total amount of radioactivity in the blood glycerides is plotted against time.)

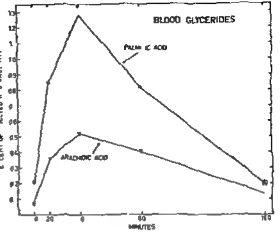

Fig 2 Blood glyceride radioactivity in fasted male rats after the intravenous injection of C arachidic acid and H palmitic acid Two rats were sacrificed at each time interval

Disappearance of label from the blood (Table I)

In both fasted and refed rats the C^{14} arachidic acid disappeared more slowly than the H^3 palmitic acid from the blood as can be seen from the increasing C^{14} to H^3 ratio in the blood free fatty acids during the first few minutes after the injection The difference is greater in the refed rats than in the fasted rats When extrapolated to zero time the disappearance curves for both arachidic and palmitic acids give values of 80 % of the injected radioactivity

Blood radioactivities at longer times in fasted rats

From 10 to 320 min after the injection the ratio C^{14}/H^3 in the cholesterol ester fraction was greater than that in the injected serum. The ratio in the free fatty acids was greater at 10 min than at 5 but after 20 min the difference gradually diminished such that at 320 min there was only half as much C^{14} label as H^3 label in the blood free fatty acids The percentage of radioactivity in blood glycerides is shown in Fig 2 There is a peak of both C^{14} label and H^3 label at 40 min The C^{14} radioactivity did not reach as high

TABLE I Ratio C /H radioactivity in the blood free fatty acid fraction in rats after the i v injection of C arachidic and H palmitic acid in rat serum The ratio in the injected fatty acids was taken as 1 0

	1	2	3	4	5	Min
Fasted	1 3	1 7	1 5	1 9	2 2	
	1 4	—	1 8	1 6	3 2	
Refed	1 9	3 6	4 3	4 0	5 0	
	1 7	2 1	3 9	6 [illegible]	—	

TABLE II Ratio C^{14}/H^{3} radioactivity in tissue lipids from rats after the intravenous injection of C^{14} arachidic acid and H^{3} palmitic acid in rat serum Each figure represents the mean of values from 3 rats ± S E M The ratio in the injected fatty acids was taken as 1 0

	Fasted		Refed	
	2 min	320 min	2 min	320 min
Liver total	0.88±0.03	0.60±0.01	0.93±0.04	0.88±0.04
Glycerides + CE	1.12±0.04	0.74±0.04	1.10±0.06	0.87±0.06
Phospholipids	0.42±0.02	0.59±0.01	0.54±0.04	0.99±0.08
Muscle total	0.88±0.09	0.62±0.01	0.72±0.04	0.72±0.08
Heart total	1.31±0.10	1.17±0.01	0.81±0.04	0.96±0.07
Kidneys total	0.66±0.03	0.32±0.01	0.47±0.03	0.38±0.02
Lungs total	1.30±0.04	0.42±0.02	0.78±0.09	0.42±0.01
Spleen total	2.57±0.05	1.44±0.09	2.09±0.07	1.98±0.10
Adipose tissue total	0.62±0.07	0.36±0.03	0.75±0.03	0.88±0.04
Carcass total	1.01±0.02	0.58±0.02	0.89±0.07	0.69±0.01
In the rat total	0.36±0.07	0.63±0.03	0.91±0.01	0.73±0.01

values as did the H^{3} radioactivity During the time period studied there was a gradual rise in both C^{14} and H^{3} label in the blood phospholipids and the percentage of arachidic acid label was greater than palmitic acid throughout

Tissue radioactivity (Table II)

2 min after injection the ratio of labeled arachidic acid to labeled palmitic acid in the fasted rats was smaller than the ratio in the injected material in the liver muscle tissue kidneys and adipose tissue and at 320 min the ratio had fallen further in all tissues

In the refed rats at 2 min there was less C^{14} label than H^{3} label in all the organs except in the spleen but at 320 min the ratio had not decreased in all the organs to the same extent as in the fasted rats The ratio of arachidic acid label to palmitic acid label was the same at 320 min as at 2 min in the muscle tissue and was greater in the heart and the adipose tissue

In the livers of both fasted and refed rats the glycerides had a C^{14}/H^{3} ratio that was greater than that in the injection solution at 2 min and smaller at 320 min In the phospholipids the ratio was less than that in the injected material at both 2 and 320 min Furthermore it was greater at 320 than at 2 min With the free fatty acids in the liver were associated about 4 percent of the injected labeled arachidic acid and almost none of the labeled palmitic acid The values were remarkably similar in the two nutritional states

Discussion

In normal blood free fatty acids are not only associated with albumin but also with other proteins and with red corpuscles The long-chain fatty acids are more bound to lipoproteins than are the short-chain fatty acids Goodman and Shafrir 1959 Th

electrophoreses of the injection solution used in the present study also indicated that more of the arachidic acid label had moved with the globulins than with the albumin. Most of the palmitic acid label moved with the albumin.

Disappearance of label from the blood

The possibility that different fatty acids are extracted from blood at different rates has been discussed earlier (Goransson and Olivecrona 1965). In the present study the arachidic acid was extracted by the tissues at a lower rate than was the palmitic acid. If this was due to the difference in the physical state in which the two acids were present in the injection solution is not clear.

Oxidation, interconversion and esterification

In spite of the lower extraction rate, labeled arachidic acid was found in slightly smaller amounts after 2 min in the total lipids both in fasted and refed rats. At 320 min, when the blood free fatty acid radioactivity had reached a value close to zero and all the injected arachidic acid thus become available for oxidation, the ratio C^{14}/H^{3} had fallen considerably both in the whole rat and in the different tissues in the fasted rats.

Arachidic acid may be converted to other fatty acids. No radioactivity was however found in the unsaturated fatty acids of the liver (Elovson, to be published). Formation of stearic acid and palmitic acid by shortening of the carbon chain would cause a loss of radioactivity in the experiments performed. The significance of this pathway can not be assessed from the present data.

It is known that after entry into the cells palmitic acid is incorporated to a varying degree into glycerides and phospholipids (Havel, Felts and van Duyne 1962, Olivecrona 1962). In the present experiments less than 0.5 % of the injected palmitic acid was recovered as free fatty acid in the liver. A higher percentage (4) of the arachidic acid was found unesterified. If this difference in esterification rate is due to the difference in the physical state in which the 2 fatty acids were present in the injection solution or in specificity in the enzyme systems in the liver is not known.

A total of 80 of the arachidic acid label found in the liver was recovered in esterified form. Initially arachidic acid appeared to be specifically incorporated into the triglycerides. Less arachidic than palmitic acid was incorporated into the phospholipids. This difference was not so marked 5 hrs after the injection.

Recirculation of label back to plasma

Blood triglycerides and blood phospholipids are known to originate in the liver (Fishler *et al.* 1943, Harper, Neal and Hlavacek 1953). When labeled oleic and palmitic acid were simultaneously injected, approximately the same ratio of oleic to palmitic acid label was found in the liver glycerides and the blood glycerides (Goransson and Olivecrona 1965). In the present experiments with arachidic acid and palmitic acid, no such similarity was observed. The difference between the C^{14}/H^{3} ratio in the liver phospholipids and the blood phospholipids was also marked.

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Miss Irene Kalbsson gave skilful technical assistance.

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From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

External Intercostal and Phrenic α Motor Responses to Changes in Respiratory Load

By

M CORDA[1] G EKLUND AND C v EULER

Received 9 July 1964

Abstract

Corda M G Eklund and C v Euler *External intercostal and phrenic α motor responses to changes in respiratory load* Acta physiol scand 1965 *63* 391—400 — The hypothesis that the fusimotor — muscle spindle system in its control of physiologically induced movements automatically adjusts the α motor activity to achieve "wanted" length changes in spite of some variations in load that may occur has been examined for the respiratory movements of external intercostal muscles and the diaphragm after bilateral vagotomy in cats under light pentobarbital anaesthesia The afferent activity from intercostal muscle spindles increased in response to an increase in respiratory load produced by tracheal occlusion Efferent intercostal α motor activity was reflexly enhanced in response to such an increase in load an effect which was abolished after section of the dorsal roots of the same and adjacent segments The efferent phrenic α motor activity and the electrical activity of the diaphragm did not show this response to tracheal occlusion indicating a principal difference in proprioceptive control of the external intercostal muscles and the diaphragm

Problems concerning the role of muscle spindles and their γ motor innervation in the control of movements may advantageously be studied on muscles which show physiologically induced movements aiming at definable length changes even during experimental conditions In anaesthetized and decerebrate preparations the respiratory movements are the only ones that fulfill these requirements An analysis of the motor control of respiration may throw some light on principles valid also for other motor systems since recent work indicates that the intercostal muscle spindles and their γ motor innervation are engaged in the control of the intercostal α motoneurones and the respiratory movements of the thoracic cage (Critchlow and Euler 1962 1963 Eccles Sears and Shealy 1962 Eklund Euler and Rutkowski 1963 a b 1964 Euler and Fritts 1963 Sears 1963)

Descending signals from the respiratory integrating mechanisms in the medulla convey the central "demand" for tidal volume in terms of changes in *length* of the individual respiratory muscles while the muscular effects produced by these signals

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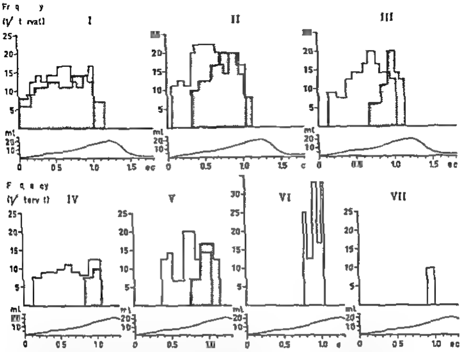


Fig 3 Same experiment as Fig 2 Here the frequency curves of the individual α fibres are shown Stippled area unrestrained spontaneous inspiration. Line in full first inspiratory effort after occlus on of the airways

den obstruction of the airways (lines in full) The immediate response to the closure of the tracheal cannula was a marked increase in the total α motoneurone activity (Fig 2) Fig 3 shows that this increase is achieved in several different ways a) by earlier engagement of the motor cells (spike II—V) b) by an increased discharge rate though only to a small extent (spike I III IV and V) and c) by recruitment of new fibres (spike VI and VII) Fig 2 and 3 further show that the increased load of the inspiratory muscles caused by tracheal occlusion led to a shortening of the duration of the total inspiratory activity as compared with that of unrestricted inspiration.

This curtailment of the inspiratory activity has been the most common response but unchanged and even prolonged inspiratory activity has been observed during tracheal occlusion The reflex increase of the activity in response to an increase of short duration in respiratory load often showed potentiation lasting for one or two breaths after the release of the obstruction This effect occurred even if the occlusion was so brief that no significant asphyxiation could develop This is illustrated in Fig 4 where A B and C are consecutive records of inspiratory activity and intratracheal pressure. The time and duration of the inspiratory flow of air has been marked Only the first inspiration in B was performed against a closed airway In this case the subsequent potentiation of the inspiration lasted during two released inspirations.

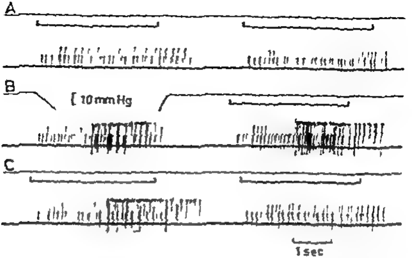


Fig 4 Inspiratory efferent α activity from a branch of the external intercostal nerve to show the effect of tracheal occlusion A–C consecutive records Upper tracing represents intratracheal pressure. The duration of inspiratory flow of air in each unrestrained spontaneous breath has been marked Tracheal occlusion only during the first inspiratory effort in B

No significant increase in the γ motor activity has been observed in response to increased respiratory load confirming previous results Eklund Euler and Rutkowski 1964

The afferent response of external intercostal muscle spindles to tracheal occlusion

Critchlow and Euler 1963 observed the marked initial increase in the inspiratory muscle spindle discharge in response to closure of the tracheal cannula at the end of the expiratory phase In the present work the response of external intercostal muscle spindles during tracheal occlusion was reexamined in 5 cats after bilateral vagotomy Fig 5 shows the considerable augmentation of the spindle discharge that is typically obtained by airway occlusion This effect indicated that the spindle is less unloaded by the shortening of the muscle body during tracheal occlusion than in the unrestricted condition This conclusion is based on the finding stated above that the fusimotor activity is largely uninfluenced by the airway closure Eklund Euler and Rutkowski 1964 Similarly it was found that some muscle spindles of the diaphragm increased their discharge rate when the movements of the diaphragm was impeded by tracheal occlusion Corda Euler and Lennerstrand 1965

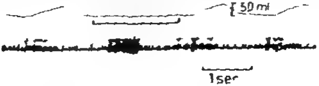


Fig 5 Afferent discharge from an external intercostal inspiratory muscle spindle to show the response to tracheal occlusion. Upper tracing represents tidal volume

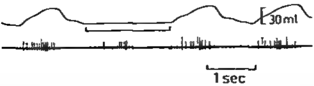


Fig. 6. Inspiratory efferent activity from a thin filament of the central end of the cut C_5 branch of the left phrenic nerve to show the response to tracheal occlusion (mark) after bilateral vagotomy. Upper tracing represents tidal volume (upward deflection denotes inspiration).

The response of phrenic motoneurones to tracheal occlusion

It has been suggested on histological grounds that the diaphragm is supplied with comparatively few proprioceptors (Dogiel 1902; Gregor 1904; Masumoto 1934 a, b). This observation has recently been confirmed with neurophysiological technique: Glebovskii (1962), Yasargil (1962) and by Corda, Euler and Lennerstrand (1965). In view of such results it was of interest to study in vagotomized preparations the effect of tracheal occlusion on the efferent phrenic activity and compare it with the intercostal response under similar circumstances, as described above. In 5 cats efferent activity was recorded from fine filaments of the central end of the cut C_5 phrenic root.

The general properties of the discharge pattern of phrenic motoneurone need not be reported here; they have been extensively described, at first in the classical paper by Adrian and Bronk (1928), later on by Pitts (1942, 1943) and recently again by Gill and Kuno (Gill, 1963, also for references; Gill and Kuno, 1963 a, b), and bear close resemblance to the intercostal motoneurone activity (cf. p. 393). In the present work, however, it was found that in response to an occlusion of the tracheal cannula in end-expiration, phrenic motoneurone activity of the next inspiration was never augmented after bilateral vagotomy as was the intercostal activity. If anything, this procedure caused a slight decrease in phrenic activity as depicted in Fig. 6.

Possibly cutting the phrenic root from which the efferent activity was recorded might have eliminated the afferent necessary for a reflex augmentation. In order to study the reflex effect of airway occlusion without impairing the afferent phrenic path the electromyogram of the diaphragm was recorded in 6 cats. In vagotomized preparations no augmenting effects were recorded in response to tracheal occlusion; there

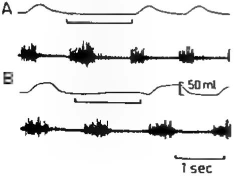
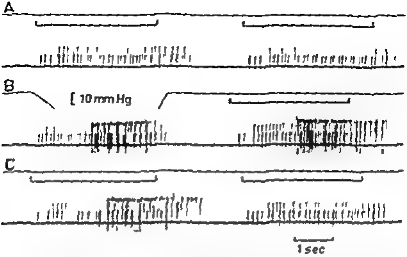


Fig. 7. Electromyogram taken from the diaphragm to show the effects of tracheal occlusion (mark). A, intact vagus nerves; B, after bilateral vagotomy.



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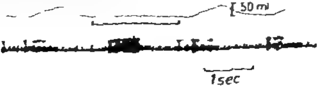

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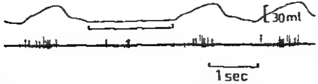

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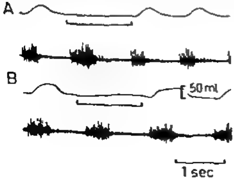

Fig. 7. Electromyogram from the diaphragm to show the effects of tracheal occlusion (mark). A, intact vagus nerves; B, after bilateral vagotomy.

Fig 8 Electromyograms from external intercostal muscle *EI* (middle tracing) and from diaphragm *D* (lower tracing) to show the responses to tracheal occlusion (mark) in the vagotomized preparation Upper tracing tidal volume

was in most cases only a slight decrease With the vagus nerves intact however a marked increase in the phrenic motoneurone activity and in the electromyogram from the diaphragm was always obtained as reported for the rabbit by Cuénod (1961) These effects before and after bilateral vagotomy are illustrated in Fig 7 Simultaneous electromyograms from the external intercostal muscles of the 6th or 7th intercostal space invariably showed a marked augmentation of the electrical activity corresponding to the effects described above from the efferent intercostal nerve twigs This is illustrated in Fig 8 which underlines the distinct difference in the proprioceptive control of the 2 main inspiratory muscles the diaphragm and the external intercostal musculature

Discussion

The present results obtained in a spontaneously moving system support the hypothesis that the autogenetic reflex effect of a load opposing a contraction of a muscle is similar to that of stretching the muscle This load compensatory reflex probably plays a fundamental role for the dynamic control of movements just as the stretch reflex does in the static control of posture The intercostal muscle spindle control system thus seems to act as a follow up length servo (Hammond Merton and Sutton 1956) which automatically tends to correct the length changes of the muscle fibres in each respiratory movement to match the central demand for volume changes in spite of variations in the load on the muscles The cat s diaphragm however does not seem to be subject to the same autogenetic length correcting adjustment to variations in airways resistance This is in general agreement with the results of Cuénod (1961) obtained in the rabbit The latter author however observed a slight augmentation in response to a partial block of the airways Nevertheless our results constitute an interesting and important difference between the control of the external intercostal muscle and the diaphragm and invites the question why such a control mechanism is so well developed in only one of the two important inspiratory muscles while it is very weak or absent in the other changes in airways resistance put similar loads on both the diaphragm and the intercostal musculature Such fundamental difference in motor control might only be expected if the two muscles were normally subjected to completely different types of variations in load This is the case in that the intercostal musculature has both respiratory and postural function so that postural movements may interfere with the respiratory function of the muscles (Masson Meulders and Colle 1960

Meulders Massion and Colle 1960) Hence movements and alterations in posture of the thoracic region cause considerable variations in the load uncountered by the respiratory movements of the intercostal muscles The diaphragm does not have such a dual function at least not to the same extent

It is interesting to note that the only effect of loading the diaphragm by means of tracheal occlusion that was observed was a slight inhibition Autogenetic inhibition seems to dominate the proprioceptive control of the diaphragm in many experimental conditions (Dolivo 1953 Gill and Kuno 1963 b) Hoffman and Keller (1929) were not able to demonstrate a definite stretch reflex from the diaphragm and Glebovskii and Pavlova (1963) found mainly inhibitory reflex reactions of the diaphragm after vagotomy An increase in the force of contraction of the diaphragm as the result of tracheal occlusion in expiratory position should not be taken as evidence of increased motor activity for it may be due to the fact that the contraction occurs during maintained stretch as is known with the same efferent activity the force of contraction of a muscle increases with increasing length The electromyogram on the other hand provides an index of motoneurone activity alone and is not directly but only reflexely influenced by changes in muscle length Cuénod s observation (1961) on vagotomized rabbits that partial obstruction of the airways caused a slight increase in the phrenic motoneurone activity while total occlusion often caused inhibition suggests that the balance between autogenetic facilitation and autogenetic inhibition may shift in accordance with the prevailing conditions the difference in mechanical threshold between spindles and tendon organs being one possible reason for such a change in balance alterations in fusimotor activity being another

When the vagal nerves were left intact tracheal occlusion always caused a definite increase in the efferent phrenic activity and in the electromyogram from the diaphragm This demonstrates that a central mechanism capable of compensating for changes in airway resistance exists in the vagal reflex circuit and in this case similarly for the diaphragm and the intercostal musculature

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Decrease in Cardiac Activity by Carotid Sinus Baroceptor Reflex

By

PERCY LINDGREN and JOHN MANNING[1]

Received 11 July 1964

Abstract

Lindgren P and J Manning *Decrease in cardiac activity by carotid sinus baroceptor reflex* Acta physiol scand 1965 *63* 401—408 — Electrical stimulation of the sinus nerve or excitation of the carotid baroceptors by increasing the intrasinus pressure significantly decreased the right ventricular contractile force heart rate and blood pressure in 19 anesthetized cats The decreases in force and rate but not blood pressure were abolished by stellate ganglionectomy and by 3—6 mg/kg of dichloroisoproterenol (DCI) The decreases in cardiac contractility and rate were not secondary to the hypotension for they were still obtained when aortic pressure was kept constant The reductions in contractile force and heart rate are the results of an inhibition of sympathetic control of the heart activity The data indicates that 40 per cent of contractile force measured in the anesthetized vagotomized preparation is dependent upon tonic sympathetic impulses In experiments in which the stellate ganglia were decentralized the force and rate were increased to predenervation levels by stimulating the isolated right stellate ganglion at a frequency of 1/sec Seventy five per cent of the maximum response in force and rate were obtained at stimulus frequencies of 3 sec The sinus baroceptor reflex exercises its major effects on the heart and blood vessels within a relatively limited range of 40 mm Hg on either side of normal blood pressure for the cat

The vasomotor events that ensue during baroceptor reflexes have been carefully studied for many organs (Heymans and Neil 1958) However there is some doubt as to the direction of change if any in cardiac activity or cardiac output that accompany these events A number of investigators have employed a variety of techniques to measure cardiac output during sinus nerve stimulation but have failed to find a consistant change (Holt *et al* 1946 Kenney *et al* 1951 and Stella 1931) Daly and Daly (1959) have reported a reduction in cardiac output during carotid sinus nerve activation but felt it was related to the reflex systemic hypotension or the bradycardia and not to a direct action on the heart In another report Daly and Luck (1958) calculated a reduced ventricular stroke work from measurements of pulmonary flow and pressure They interpreted these findings as evidence for a decreased right ventricular contractility but it was not a consistant finding during elevation of carotid sinus pressure Manning *et al* (1963) have recently shown that depressor responses elicited from the septal region of the cat results in an inhibition of tonic cardiac sympathetic activity and thereby a reduction in heart force and rate The present experiments were designed

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TABLE I Average depressor responses elicited by electrical stimulation of the left sinus nerve gangha and 3—6 mg/kg DCI on the responses

	Response to sinus nerve stimulation					
	Number of obs	Untreated Control	Response	Number of obs	During aorta occlusion Control	Response
Number of animals		11			11	
Right ventricular contractile forcegram	38	34 ±[illegible]3	26 ±1 8	20	39±3 3	30±2 7
Heart rate beats/min	38	177 ±3 7	155 ±3 1	20	174±3 9	151±4 1
Blood pressure mm Hg	38	136 ±2 8	93 ±4 9		Held constant	
Blood flow ml/min	38	8 6±0 6	6 2±0 5		Not recorded	

Values are means ± S D Significantly different from corresponding control value in that *Significantly different from corresponding control value in group untreated (0 02*

1) to measure the changes in cardiac contractile force and heart rate during elevation of sinus pressure or stimulation of the sinus nerve 2) to determine the mechanisms responsible for these changes and 3) to evaluate the quantitative control the sinus roceptor reflex exercises on cardiac force and rate

Methods

Successful experiments were performed in 23 cats of both sexes ranging in weight from 2 1 to 4 4 kg The animals were anesthetized with 50 to 70 mg/kg of α-chloralose i v Artificial ventilation was provided with room air using a Palmer pump to deliver a tidal volume of 30—45 ml at a respiratory rate of 16—25/min Body temperature was monitored by a thermometer inserted in the rectum and was kept between 36—37 C by means of heat lamps Arterial pressure was measured with a Statham P 23 AC transducer connected to a catheter placed in a femoral or common carotid artery Ventricular contractile force was measured with a strain gauge arch sutured to the right ventricle Right ventricular contractile force was measured because varying systemic pressures have relatively less effect on the right than on the left ventricular end-diastolic fiber length (Cotten and Bay 1956 and Rosen 1961) Blood flow in the hind limb was measured with drop rate chamber as described by Lindgren (1958) Heart rate was determined using an interval recorder (Goldschmidt and Lindgren 1961) The measurements of pressure force rate and flow were synchronously recorded on a Grass polygraph Heparin (25 mg/kg) was given i v in those experiments in which blood flow was recorded To circumvent the vagal action on the heart the vagus nerve was cut bilaterally in 17 experiments while in 6 cats 0 75 mg/kg of atropine was given i v The carotid sinus nerves were dissected free and a pair of silver stimulating electrodes placed on the left sinus nerve The nerve-electrode preparation was covered with warm paraffin oil In 8 animals the carotid sinus region was isolated from the circulation and perfused with blood from a pulsating pump The pump action was produced by three pistons acting on a rubber tube two served as valves in the inflow-outflow circuit Pressure was developed by the third piston by compressing a section of the tube The output of the pump varied depending on the resistance to flow so that at 50 mm Hg a flow rate of 16 ml min was obtained while at 780 mm Hg the flow rate was

preparation and the effects of partial occlusion of the thoracic aorta extirpation of the stellate

Number of ob	After stellatectomy Control	Response	Number of obs	After 3–6 mg/kg DCI Control	Response
	8			3	
8	21 ±3 3	20 ±3 3	17	' 24 ±2 3	23 ±1 9
8	135 ±7 7	132 ±8 1	17	157 ±2 2	155 ±2 6
8	103 ±9 5	86 ±9 3	17	109 ±4 1	66 ±3 4
8	3 9±0 3	2 2±0 2	7	3 6±0 3	? 5±0 2

group (0 0? > P > 0 001)
P ~ 0 001)

1 ml/min The animal served as its own blood donor and blood was led from the proximal common carotid artery to the pump From the pump blood perfused the sinus region via twin catheters which were ligated in the common carotid just proximal to the sinus The blood was returned to the animal from the external carotid via the left jugular vein All other vessels in the sinus region were ligated The pump's valve action was such that there was no through communication of pressure from the animal to the sinus area during the inflow or outflow cycle A pulse amplitude was realized by varying the size of stroke of the pumping piston but the frequency of the pump cycle was fixed at 63/min The mean pressure in the perfusion circuit could be raised to any desired level by adjusting a resistance clamp on the catheter returning blood to the jugular vein

In 4 animals the sinus nerves the vagal nerves and the preganglionic roots of both stellate ganglia were severed A pair of stimulating electrodes were placed on the denervated right stellate ganglion and covered with paraffin oil The stimulus consisted of a train of square wave pulses derived from a Grass model S 4 stimulator and isolation unit. The sinus nerve was stimulated at a frequency of 30 cycles/sec, a pulse duration of 5 msec at an intensity sufficient to produce a maximum fall in blood pressure The stellate ganglia were stimulated with an intensity of 2 5 to 3 0 V of 5 msec duration at various frequencies

Results

Electrical stimulation of the carotid sinus nerve produced the predictable fall in systemic pressure with a concomitant decrease in cardiac force and rate These events together with a reduction in limb blood flow appeared within a few seconds after onset of stimulation and could be elicited repeatedly In order to be uniform in the experimental procedure the left sinus nerve was selected for 1 min of stimulation The average response from 11 expts in force rate pressure and flow are presented in Table I in the first column labeled untreated The magnitude of the depressor action was similar upon repeated stimulation in the same experiment and from one experiment to another No efforts were made to obtain graded responses by altering the stimulus

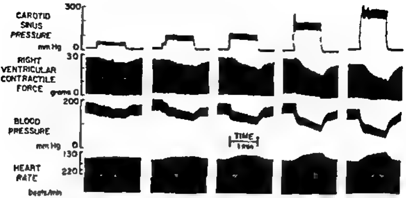

Fig 1 Decreases in cardiac contractile force heart rate and blood pressure by step-wise increases in perfusion pressure of both right and left isolated carotid sinus Intrasinus pressure was raised for one minute from ?0 mm Hg to the levels shown by adjusting a resistance clamp on the catheter returning blood from the sinus region to the jugular vein The first detectable decrease in force rate and pressure was noted at intrasinus pressures as low as 30—40 mm Hg Both vagus nerves were cut and the sympathetic nerves remained as the only functional innervation to the heart Cat ? 4 kg fi

parameters On the average the sinus nerve stimulation elicited a sizeable per cent change in contractile force (24 %) and blood pressure (37) The reduced blood flow in the hind limb was secondary to the blood pressure fall in for 3 expts in which the perfusion pressure was kept constant by cross-circulation with a donor animal there was an increase in blood flow during sinus nerve stimulation

The possibility that the lowered arterial pressure in the responses described above brought about the fall in cardiac force and rate was tested in the same 11 preparations An adjustable clamp was placed around the thoracic aorta in such a manner that the aortic pressure proximal to the clamp was held constant during the reflex evoked depressor response The results are presented in Table I in the column labeled during aortic occlusion When aortic pressure was kept at or near control levels stimulation of the sinus nerve still significantly decreased contractile force and heart rate showing that the observed cardiac effects were not secondary to the blood pressure fall

To demonstrate that the cardiac depression seen in these reflex responses was dependent upon adrenergic innervation of the heart the stellate ganglia were removed in 8 expts and a beta adrenergic blocking drug was administered in 3 Removal of the stellate ganglia decreased the cardiac contractile force to 33 per cent below the control "untreated level (Table I) The inhibitory reflex evoked by electrical stimulation of the left sinus nerve brought about 2/3 of the reduction in cardiac force that was caused with complete removal of sympathetic impulses by stellate ganglia extirpation Heart rate was also markedly reduced by removal of the stellate ganglia Sinus nerve stimulation after ganglionectomy had no effect on contractile force and heart rate but the characteristic fall in blood pressure was still produced The beta adrenergic blocking drug dichloroisoproterenol (DCI) also prevented the decrease in cardiac force and rate induced by sinus nerve stimulation but like stellate ganglionectomy it did not prevent the fall in blood pressure Table I) The dose of DCI employed was large and reduced contractile force and heart rate to levels between those reached

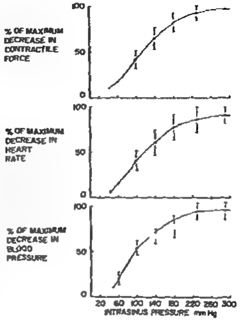

Fig 2 The relationship between sinus pressures and reflex decreases in cardiovascular activity measured in 8 animals treated as in Fig 1 The largest decrease in force rate and pressure was taken as the maximum response whether or not this occured at the highest intrasinus pressure or below The mean responses at a given sinus pressure are presented as the per cent of the maximum decrease ± S D The 50 per cent level of the possible reflex effect was reached at sinus pressures of 100—120 mm Hg

with ganglionectomy plus by activation of the sinus reflex in the untreated group These experiments also support the idea that a fall in systemic blood pressure does not give rise in itself to a reduction in contractile force of the right ventricle The blood pressure falls were substantially as large as in the untreated group but no changes were seen in contractile force and heart rate after the nerve supply to the heart had been blocked

In Fig 1 are shown the qualitative effects of the carotid sinus baroceptor reflex on the heart which has the sympathetic nerves as its only functional innervation In this and 7 other expts the isolated carotid sinus region was perfused by a pulsatile flow of blood As the intrasinus pressure was raised for 1 min in graded steps the blood pressure heart rate and contractile force decreased progressively with each increment in sinus pressure

The data from 8 animals so treated were grouped and described the curves shown in Fig 2 The decreases in each parameter with increased intrasinus pressure were plotted as the per cent of maximum change ± S D The first detected decrease in force rate and pressure was noted at a mean intrasinus pressure of 30 to 40 mm Hg At sinus pressure of 60 to 140 mm Hg the decreases in cardiac rate and force were nearly proportional to the increases in intrasinus pressure The reflex effects on force rate and pressure approched maximum activity above sinus pressure of 180 mm Hg so that the slopes of the curves markedly decreased

The range of control exercised on cardiac contractile force and rate by the heart's sympathetic innervation was studied in 4 animals by reversing the experimental procedure Having sectioned the vagus nerves the preganglionic supply to both stellate ganglia was cut The right ganglion was selected for stimulation because of the position

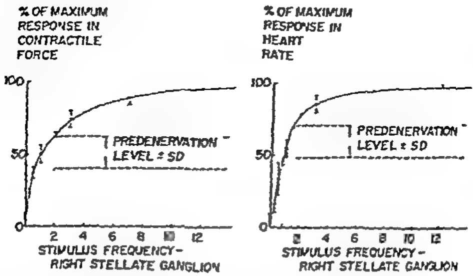

Fig. 3 Responses in contractile force and heart rate to stimulating the decentralized right stellate ganglion at various frequencies. From 4 cats the average response ± S.D. at a given frequency of stimulation was computed as a per cent of the maximum response for each animal. In the beginning of the experiments the vagus nerves were cut. The preganglionic roots of both stellate ganglia were sectioned and the decreases in contractile force and heart rate were measured. The level so obtained corresponds to the zero level in the diagrams. The average value ± S.D. from 4 cats before decentralization of the ganglia but after vagotomy is represented by the shaded areas in the diagrams as well as the range in stimulus frequency needed to re-obtain these levels.

of the animal in these studies. The ganglion was stimulated at frequencies in the range of 0.25 to 20/sec. The increase in force and rate elicited by a given frequency of stimulation was expressed as a per cent of the maximum response for that animal. In Fig. 3 the average responses ± S.D. are plotted as a function of the stimulus frequency. The exponential curves so obtained illustrate the relatively low frequencies of stimulation needed to effect a large increase in cardiac rate and force. The shaded areas in the graphs represent the level of force and rate present before the preganglionic roots were sectioned. This level ± S.D. in force and rate was reached by stimulating the right stellate ganglion at frequencies of 2/sec or less. Stimulus frequencies above 3/sec increased contractile force and heart rate proportionally less than stimulus frequencies below this value.

Discussion

The decrease in cardiac contractile force and heart rate elicited by carotid sinus nerve stimulation appears to be the result of a reduction of tonic sympathetic activity. This interpretation is supported by the data from those experiments in which the stellate ganglia were removed and in which a beta adrenergic blocking drug (DCI) was given. Acute extirpation of the stellate ganglia reduced contractile force and heart rate to levels lower than reached during sinus nerve stimulation before ganglionectomy and

sinus nerve stimulation after ganglionectomy did not reduce the force and rate further The beta adrenergic blocking drug DCI also effectively prevented the decrease in force and rate during sinus nerve stimulation The reduction in contractile force and heart rate after DCI was somewhat smaller than that after ganglionectomy If such a difference is real it may have resulted from the direct stimulant action of DCI on the heart Nevertheless DCI has been shown to prevent not only the cardiac responses to catecholamines but also the responses to electrical stimulation of the cardiac sympathetic nerves (Moran and Perkins 1958)

In contrast to the effects of ganglionectomy and DCI on the cardiac responses to sinus nerve stimulation the characteristic fall in blood pressure was as expected not altered The reduction in blood pressure which is part of the reflex response induced by activation of carotid sinus baroceptors was not in itself responsible for the decrease in force and rate The latter were readily obtained in the absence of a fall in mean systemic pressure that the heart must work against as was demonstrated when aortic mean pressure was kept constant during sinus nerve stimulation by means of aortic compression Thus a reduction in sympathetic discharges to the heart would decrease the cardiac contractility by placing the heart on a different force tension curve Also under these conditions it is not likely that there would be a significant reduction in coronary blood flow which Heymans and Neil (1958) suggested would account for a diminished mechanical efficiency of the ventricles

The important role this reflex mechanism plays in regulating cardiac contractile force heart rate and blood pressure by inhibiting tonic sympathetic activity was manifested under the more physiological conditions of activating the baroceptors by perfusing the sinus region with a pulsatile blood flow As shown in Fig 2 50 per cent of the maximum reduction in force rate and pressure was obtained by increasing the intrasinus pressure from 20 to 120 mm Hg This is the usual value given for the mean arterial pressure of an anesthetized cat Indeed a sinus pressure of 80 mm Hg produced about 30 per cent of the maximum reduction in the three parameters and 75 per cent is reached already at 160 mm Hg These values represent the steepest and most linear portions of the curves where a minor change in sinus pressure means a marked adjustment in sympathetic control It is apparent that under conditions of these experiments the sinus reflex exercises its major effects within the limited range of 40 mm Hg on either side of normal blood pressure for a cat

As seen in Table I the average predenervation level of blood pressure in our series was 136 mm Hg According to the conclusions drawn above from Fig 2 this would mean that in these cats the tonic sympathetic activity was between 50 to 60 per cent of the maximum influence exerted on cardiac function In the experiments where the stellate ganglia were decentralized and then stimulated at various frequencies the level of nervous influence existing in the resting anesthetized cat could be determined Thus in Fig 3 the predenervation level lies at about 50 per cent of the maximum response in cardiac force and at 60 per cent in heart rate where the minimum was taken as the denervated level and the maximum was the change at supermaximum stimulation of the right stellate ganglion It is hard to say if this difference between the rate and force curves is real However the rate curve is steeper for 2/sec stimulation gives an 80 per cent response in rate but only 62 per cent in force Such a difference may be accounted for if the fibers controlling the chronotrophic function are predominantly derived from the right ganglion In fact Randall and Rohse (1956) have presented functional evidence that such a condition exists in the dog

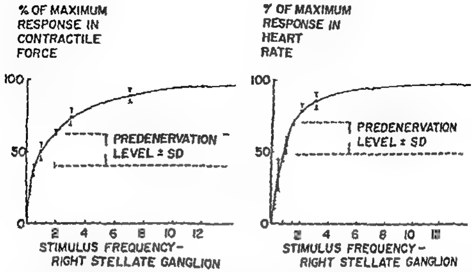

Fig 3 Responses in contractile force and heart rate to stimulating the decentralized right stellate ganglion at various frequencies From 4 cats the average response ± S D at a given frequency of stimulation was computed as a per cent of the maximum response for each animal In the beginning of the experiments the vagus nerves were cut The preganglionic roots of both stellate ganglia were sectioned and the decreases in contractile force and heart rate were measured The level so obtained corresponds to the zero level in the diagrams The average value ± S D from 4 cats before decentralization of the ganglia but after vagotomy is represented by the shaded areas in the diagrams as well as the range in stimulus frequency needed to re obtain these levels

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Discussion

The decrease in cardiac contractile force and heart rate elicited by carotid sinus nerve stimulation appears to be the result of a reduction of tonic sympathetic activity This interpretation is supported by the data from those experiments in which the stellate ganglia were removed and in which a beta adrenergic blocking drug (DCI) was given Acute extirpation of the stellate ganglia reduced contractile force and heart rate to levels lower than reached during sinus nerve stimulation before ganglionectomy and

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Synaptic Influence on the Repetitive Activity Elicited in Cat Lumbosacral Motoneurones by Long-Lasting Injected Currents

By

DANIEL KERNELL

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Long lasting repetitive discharges can be elicited in motoneurones by steady currents injected through the tip of an intracellular microelectrode (e g Granit Kernell and Shortess 1963 *a b* Shapovalov 1964) In such discharges the relation between firing rate and strength of stimulating current (the "f—I relation") is linear over a considerable range The proportionality constant (the "f—I slope") of the linear f—I relation decreases during a brief (less than 1 sec) initial phase of adaptation but is thereafter constant for long (up to more than 1 min) periods of continuous repetitive firing (Granit *et al* 1963 *a b*) The f—I slope which is obtained from repetitive discharges after the initial phase of adaptation will be referred to as the "steady state f—I slope"

The present study deals with the possible synaptic influence on the value of the steady state f—I slope of cat lumbosacral alpha motoneurones The cats were anaesthetized by pentobarbitone (35—40 mg/kg i p) Values of steady state f—I slopes were obtained from long lasting repetitive discharges elicited by currents injected through the tip of an intracellular microelectrode (*cf* Granit *et al* 1963 *a b* Fig 1 is a diagram showing the steady state f—I slopes for one motoneurone Slope *a* (Fig 1) is before slope *b* ■ during and slope *c* is after a fast (740 sec) tetanic stimulation of the brain stem in the region of the red nucleus ipsilateral to the recording site Brain stem stimulation caused a steady decrease of the membrane potential by about 3 0 mV The steady state f—I slope obtained during stem stimulation (line b 3 3 imp/sec/nA) was greater than the corresponding f—I slopes obtained from the control experiments (lines *a* and *c* 2 1 imp sec/nA) The difference was found to be statistically significant (*t* test $0.01 < p < 0.001$)

Similar experiments were performed in four other motoneurones In one cell tetanic stimulation of the brain stem decreased the membrane potential by about 3 5 mV and increased the steady state f—I slope from 1 9 to 3 5 imp sec nA In a second motoneurone tetanic brain stem stimulation increased the membrane potential by about 2 6 mV and increased the steady state f—I slope from 1 1 to 2 1 imp sec nA In a third motoneurone tetanic stimulation of the common peroneal nerve increased the mem

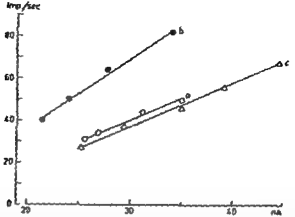

Fig 1 From a motoneurone with antidromic sp ke amplitude 73 m\ The diagram shows the relation between discharge frequency (imp/sec) and strength of stimulating current (nA 1 nA = 10 A) for long lasting repetitive firing set up by injected depolarizing currents alone (line a) by injected currents plus a tetanic stimulation of the brain stem (line b) and later on e more by injected currents alone (line c) A pause of several minutes was allowed between each of the three trials (l nes a—c) and in each trial the current strength was changed in steps D scharge frequencies were stable f r each current strength over the period of measurement (0 5 sec) Regression l nes were calculated by the method of least squares

brane potential by about 1 5 mV and decreased the steady state f—I slope from 4 4 to 3 5 imp/sec/nA In these three cells the change in steady state f—I slope was statis y significant. In a fourth cell tetanic stimulation of the hamstring nerve decreased membrane potential by about 3 4 mV but did not affect the steady state f—I lope There was no indication that the present results were due to inaccurate mea surements

It may be concluded that steady synaptic action can alter the value of the steady state f—I slope of cat lumbosacral motoneurones Furthermore it appears as if this change of the steady state f—I slope is largely independent of whether the synaptic ac tion is inhibitory or excitatory as judged by the change of membrane potential The possible physiological significance of the synaptic influence on the steady state f—I slope is being subjected to further study

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Effect of Axon Division on the Distribution of Noradrenaline and Acetylcholinesterase in Sympathetic Neurons of the Rat

By

Olavi Eränkö and Matti Härkönen

The distribution of histochemically demonstrable noradrenaline (NA) suggests that this amine is synthetized in the cytoplasm of sympathetic ganglion cells and migrates along the axon towards the periphery (Eränkö and Härkönen 1963). To test this possibility, the effect of nerve division was examined in the present study. Besides the distribution of NA, that of acetylcholinesterase (AChE) was studied, because previous work showed that many sympathetic cells with a high NA content also exhibit an intense AChE activity (Eränkö and Härkönen 1964).

Pre- or postganglionic nerves of the superior cervical ganglion of the rat were divided a few mm from the ganglion. After a period ranging from 1 day to 3 months, the ganglion and the nerve stumps attached to it were removed and freeze-dried. NA was demonstrated with formaldehyde-induced fluorescence (Eränkö 1964) and AChE activity with Gomori's (1952) modification of Koelle's thiocholine method. Non-specific cholinesterase was inhibited with tetraisopropylpyrophosphoramide (iso-OMPA).

Division of the preganglionic nerve trunk had no marked effect on NA content or AChE activity of the ganglion cells, although it caused a virtually total loss of AChE activity from the preganglionic fibres and synapses.

Postganglionic denervation resulted in a loss of both NA and AChE activity from the ganglion cells. Very little AChE activity was seen in these cells 2 days after the operation, while the loss of NA was slower, reaching a maximum in about a week. However, accumulation of NA in the proximal nerve stump was clearly demonstrable already after [illegible] days (Fig. 1). AChE activity of the stump was also increased. The small intensely fluorescent cells normally present in the ganglion (Eränkö and Härkönen 1963) were not affected by nerve division. NA content and AChE activity of the ganglion cells gradually increased 1—2 months after denervation, reaching the normal level in about 3 months.

The observations presented indicate that NA and AChE indeed migrate from the cell body to the axon after this has been divided. Dahlström and Fuxe (1964) have in a recent study, published after the present work was completed, independently arrived

Fig 1 Distribution of formaldehyde induced fluorescence in the sympathetic ganglion and two postganglionic nerve trunks 2 days after division of the latter The fluorescence of the ganglion cell bodies (left upper corner) is weak that in the ends of the nerve stumps is extremely intense Small intensely fluorescent cells are visible in the ganglion between the nerve trunks Such cells are normally present in the ganglion 30 ×

at the same result concerning NA and made use of it to demonstrate monoamine containing fibres in the central nervous system

It appears likely that axonic flow of NA and AChE occurs not only after nerve division but also in an intact neuron However this does not exclude the possibility of their uptake or synthesis in the peripheral parts of the nerve fibre (see de Robertis 1964)

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Acta physiol scand 1965 63 413—422

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Uptake of Catecholamines by the Hearts of Rabbits Treated with Segontin

By

B R MACKENNA[1]

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Abstract

Mackenna B R *Uptake of catecholamines by the hearts of rabbits treated with Segontin* Acta phys ol scand 1965 *63* 413—422 — A single i v injection of 8 mg/kg of Segontin caused a fall in the NA content of rabbit hearts to less than 10 per cent of their normal value in about 2 hrs After 4 hrs the tissue content of NA started to rise again and after 24 hrs it had recovered to about one third of its normal value An i m injection of 0 75 mg/kg NA 2 hrs after Segontin treatment caused a temporary rise in the NA content of the heart to the normal value NA was increased in the coarse particle fraction the high speed sediment and in the particle free supernatant fraction of the heart after homogenization indicating that the exogenous NA is temporarily filling the available storage sites An i v infusion of NA (10 μg/kg min for 10 min) caused an uptake of NA into the heart previously depleted by Segontin followed by a rapid and then slower release of the NA The uptake of l NA and d NA and also of l A and d A showed stereochemical specificity in favour of the l isomers Phenoxybenzamine blocked the uptake of NA into the heart of rabbits depleted by Segontin whereas DCI had only a small blocking effect Phenoxybenzamine by itself reduced the NA content of all fractions of the heart

The drug N (3 Phenyl propyl (2)) 1 1-diphenyl propyl (3) amine (Segontin) has been shown to cause a 50—60 % decrease in the amount of noradrenaline (NA) in the brain and myocardium of rats (Schone and Lindner 1960) It releases NA from adrenomedullary granules (Schone and Lindner 1962 Carlsson Hillarp and Waldeck 1963) and from splenic nerve granules (Euler Stjarne and Lishajko 1964) The present investigation was undertaken to extend these observations by studying the effect of Segontin on different fractions of the rabbit heart after homogenization and the effect of administration of exogenous NA on the tissue stores

The uptake of catecholamines into intact tissues has previously been studied mainly in two ways Unlabelled amines have been infused or injected and the increase of the amine content of various organs above the normal value has been measured (Raab 1943 Nickerson Berghout and Hammerstrom 1950 Raab and Gigee 1955 Stromblad and Nickerson 1961 Campos and Shideman 1962)

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The other main method of studying uptake has been by the use of a tracer dose of radioactively labelled amines In these investigations it is difficult to differentiate between net accumulation and simple exchange of amines

In the present investigation the uptake of catecholamines was studied in a tissue which had been first depleted of more than 90 per cent of its amines by Segontin The NA content of the tissue and its fractions after parenteral NA administration then indicates to what degree a true NA uptake has occurred

Methods

Rabbits of either sex and usually weighing between 2 and 3 kg were used The animals were anesthetized by an i v injection of 20 % urethane and then sacrificed by cutting their carotid arteries

For estimation of the A and NA in the different fractions the hearts were cut open rinsed with 0 9 % NaCl blotted dry and weighed They were then placed in a mixture containing 32 ml of ice cold isotonic potassium phosphate buffer pH 7 5 and 2 ml of EDTA containing 200 mg/ml The hearts were cut into small pieces with scissors and then homogenized for 45 sec with an Ultra Turrax apparatus set at a moderate speed

The volume of homogenate was made up to 42 ml and 15 ml of this was extracted with 15 ml of 10 % trichloracetic acid for estimation of the catecholamines in the whole heart The other 27 ml were then separated into 3 fractions called coarse particles high speed sediment and particle free supernatant by differential centrifugation as described below

The homogenate was first centrifuged at 8 000 × g for 15 min The sediment (coarse particle fraction) was resuspended in ice cold phosphate buffer and both this suspension and the supernatant were then centrifuged at 100 000 × g for 35 min At the end of this time the particle free supernatant along with the buffer from the coarse particle tubes were extracted with 10 % trichloracetic acid as were the coarse particles and the high speed sediment

The extracts were filtered adsorbed on alumina and the catecholamines (A and NA) determined by fluorimetric analysis according to Euler and Lishajko (1961) No correction was made for the amines lost during this procedure approximately 20 %

Usually when fractionation was not carried out and only whole heart catecholamines were estimated the hearts were homogenized in ice cold phosphate buffer EDTA mixture as in the fractionation procedure and 15 ml portions were extracted with 10 % trichloracetic acid in order to keep the estimation procedure constant However in the experiments where the uptake of noradrenaline infused i v was studied the hearts were homogenized in 10 % trichloracetic acid filtered and the total catecholamines estimated as above

In the infusion experiments the animals were anesthetized with 20 % urethane and received 10 μg/kg min of NA for 20 min into the femoral vein with a slow injection apparatus The NA solution for infusion was made up to 10 ml with isotonic sodium chloride

Segontin was administered either as the gluconate or the lactate The gluconate was diluted from a 5 % solution with 6 % dextrose so that the solution for injection contained 5 mg/ml The lactate was dissolved in a small volume of propylene glycol and then diluted with 6 % dextrose to make a 5 mg/ml solution Segontin was given by i v injection into the marginal vein of the ear

The drugs used in the experiments were Segontin gluconate and Segontin lactate (Hoechst) l and d noradrenaline bitartrate l adrenaline bitartrate d adrenaline base dissolved in 0 1 N HCl 3 hydroxytyramine hydrochloride (dopamine) phenoxybenzamine hydrochloride (PBA) dichlorisoproterenol (DCI) Dr F P Luduena Sterling Winthrop Research Institute Rensselaer N Y kindly supplied highly purified d isomers of A and NA and Dr A Wegmann Hoechst the Segontin.

All concentrations of NA and A in these experiments are expressed as the base Concentrations of PBA DCI and dopamine are expressed as the salt

All salts of A and NA and dopamine for i m injection were dissolved in saline to make a concentration of 1 mg per ml DCI and PBA were dissolved in saline to make a solution of 5 mg/ml of the salt for i v injection

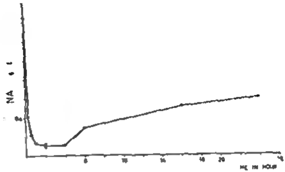


Fig 1 NA content of the hearts of rabbits at various times after a single i v injection of 8 mg/kg of Segontin Each dot in this and subsequent figures represents an estimation in one animal

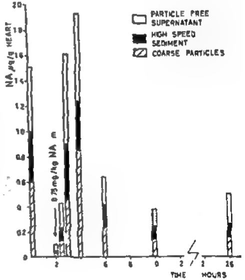


Fig 2 Time course of uptake of NA into different fractions of the hearts from Segontin treated rabbits Left hand bar shows distribution of NA in the fractions of normal hearts After 2 hrs an injection of 0 75 mg/kg NA i m causes an increase in the NA in all fractions Each bar represents the mean of 2 or 3 expts except the normal which is the mean of 24 expts

Results

Effect of Segontin on the heart NA

The NA content of the rabbit heart fell to about 8 of its original value in less than 2 hrs after a single i v injection of 8 mg/kg of Segontin (Fig 1) It remained at this low level for about 2 hrs and then slowly increased to about one third of the normal value in 24 hrs

Depletion was observed in all 3 fractions of the heart The relative proportions of NA in the different fractions remained approximately constant throughout the depletion period

There was very little A in the hearts of untreated rabbits less than 0 1 µg/g This value fell even lower to about 0 02 µg/g 2 hrs after an injection of Segontin

The dose of Segontin used (8 mg/kg) caused convulsions in the rabbits characterized by trashing of both the fore and hind limbs and hyperextension of the neck The con

TABLE I The distribution of NA (μg/g of heart M±SEM) in the fractions of the heart from I normal rabbits II normal rabbits injected with NA III Segontin treated rabbits (8 mg/kg i v) after 2 hrs injected with NA Hearts removed 2 hrs later

		Coarse particles	High speed sediment	Particle free supernatant
I	Normal rabbit n=24	0 53±0 04	0 76±0 03	0 50±0 1
II	Normal rabbit + NA 0 75 mg/kg i m. n=2	0 61±0 05	0 41±0 08	0 64±0 11
III	Segontin treated rabbit NA 0 75 mg/kg i m n=3	0 86±0 43	0 38±0 05	0 69±0 07

n=number of experiments

vulsions came on intermittently approximately every half minute to begin with but gradually with longer intervals till they passed off in a half to three quarters of an hour The animals also displayed nystagmus grinding of the teeth rapid respiration and after the end of the convulsions they were ataxic for about 1 hr The animals appeared sedated for more than 2 hrs but showed no miosis or diarrhoea or other signs characteristic of reserpine treatment About 3 hrs after the injection the animal appeared normal when the level of NA in the heart was still low Doses of less than 5 mg/kg lowered the NA levels in the tissues to a lesser degree without producing convulsions

Uptake of NA after depletion by Segontin

In these experiments rabbits were given a single injection of 8 mg/kg of Segontin at zero time After 2 hrs when the level of NA in the heart was low (about 0 1 μg/g) the rabbits were given 0 75 mg/kg of NA by i m injection The rabbits were sacrificed at various times after the injection of NA the hearts were fractionated and the NA content of each fraction was estimated

Fig 2 shows that there was a rapid uptake of NA which reached a peak 2 hrs after the NA injection Thereafter there was again a fall in the NA content to about 25 of the normal value

It is noteworthy that the NA which was taken up by the tissues was found in all fractions and was almost equally distributed between them

2 rabbits were injected with 0 75 mg/kg NA i m and their hearts were fractionated 2 hrs later These results are compared in Table I with the distribution of NA in the hearts of normal rabbits and in the hearts of Segontin treated rabbits 2 hrs after an i m injection of 0 75 mg/kg NA The table shows that while there may appear to have been an increase to above the normal in the NA content of the coarse particle and particle free supernatant fractions of both the Segontin treated and the normal rabbits injected with NA these differences are not statistically significant

Effect of dopamine on the NA content of the heart of Segontin treated rabbits

In some experiments dopamine (1 5 mg/kg) was injected i m 2 hrs after an injection of Segontin and the NA content of the heart was estimated at various times after the dopamine injection

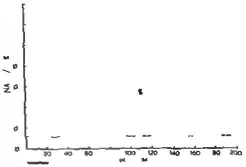


Fig 3 Concentration of NA at various times after the end of an i v infusion of NA into a Segontin treated rabbit Two hours after the Segontin injection NA was infused for 20 min (10 mg/kg min) represented by block bar) The dotted line represents the expected level of NA after Segontin treatment

Dopamine caused a rise in the NA content of the heart This reached a peak 1/2 hour after the injection and rose to about half the normal content of NA It then fell rapidly and after 4 hrs the NA content of the heart was again low

Effect of an intravenous infusion of NA

To obtain a clearer idea of the time course of the disappearance of the NA which had been taken up several rabbits were infused with NA i v The infusion was commenced 2 hrs after the Segontin injection and lasted for 20 min In this time 10 μg/kg/min of NA were infused Rabbits were sacrificed at various times after the end of the infusion and the NA content of the whole heart was estimated

As can be seen in Fig 3 the NA content of the heart was increased by the i v infusion In one rabbit which died immediately at the end of the infusion and therefore is not included 3 μg/g of NA were found in the heart After the infusion there was a rapid fall in the NA content over the first 5—10 min after which an approximately constant level was reached

The amount of NA in the heart 10 min and longer after the end of the infusion was significantly ($0.01 > P > 0.001$) above the amount which one would expect in the heart 2 hrs after Segontin treatment

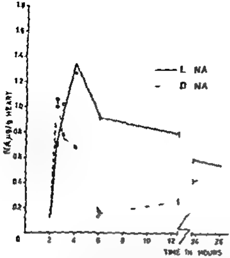
The time course of disappearance of NA from the heart under the present experimental conditions was similar to that found for tritium labelled norepinephrine by Kopin Hertting and Gordon (1962)

Uptake of l NA and d NA

Having demonstrated that l NA could be taken up by a heart which was depleted of its catecholamines by Segontin the studies were extended to see if there was any difference between the uptake of l NA and d NA

Rabbits were injected with Segontin at zero time and 2 hrs later 0 41 mg/kg of l NA or d NA was injected i m so that the uptake of NA by the tissues could be compared with the uptake of A This amount is less than that injected in the earlier experiments (0 75 mg/kg) since 0 75 mg/kg A was found to be too toxic for the rabbits The pattern of the uptake of l NA and d NA is shown in Fig 4

There was a fairly wide scatter of the uptake pattern probably because the levels were changing so rapidly However it appears that the uptake of l NA reached a peak later than d NA The uptake of l NA reached a higher level than d NA and also l NA was released more slowly than d NA



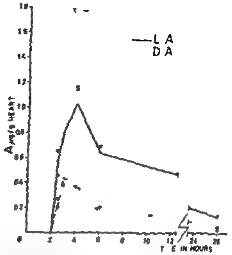


Fig 4 (Left) The uptake of l NA and d NA 0 41 mg/kg injected i m into Segontin treated rabbits. At zero time the rabbits were injected with Segontin and at 2 hrs NA was injected The level at 2 hrs is the expected NA level after Segontin treatment alone *Line drawn through means*

Fig 5 (Right) Uptake of l A and d A into the heart of Segontin treated rabbits At zero time the rabbits were injected with Segontin i.v *and at 2 hrs with l or d A i m Line drawn through means*

Uptake of l A and d A

A similar series of experiments was carried out using l A and d A The same dose (0 41 mg/kg) of l A or d A was injected i m into rabbits 2 hrs after they had been given Segontin The hearts were estimated for A at various times after this injection

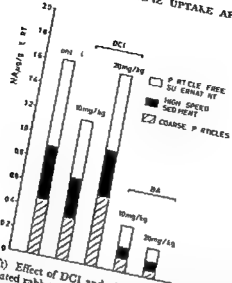
Fig 5 shows that under these circumstances at all times there was a higher level of A after the injection of l A than there was after d A

The effect of DCI and phenoxybenzamine on NA uptake

The α receptor blocking agent phenoxybenzamine (PBA) has been shown by Brown and Gillespie (1957) to increase the overflow of transmitter on stimulation of the sympathetic nerves to the perfused spleen of the cat. Since the receptors in the heart have been reported to be the same as adrenergic inhibitory receptors (β receptors) and are blocked by dichloroisoproterenol (DCI) (Moran and Perkins 1958) it was decided to establish whether this substance would block the uptake of NA into the rabbit heart

Rabbits were injected with Segontin and 1 3/4 hrs later they were injected i v with 10 mg/kg or 20 mg/kg of DCI At 2 hrs they were injected i m with 0 75 mg/kg of NA One hour later the NA content of the heart was estimated As shown in Fig 6 10 mg/kg of DCI reduced only slightly the uptake of NA and 20 mg/kg had no effect

Similar experiments were carried out with 10 mg/kg and 20 mg/kg of PBA which was injected i.v 1 hr before the i m injection of NA Fig 6 shows that PBA considerably reduced the uptake of NA into all fractions of the rabbit heart



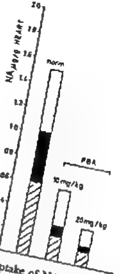


Fig 6 (Left) Effect of DCI and phenoxybenzamine on the uptake of NA into the heart of Segontin treated rabbits In all cases NA was injected 2 hrs after the Segontin treatment DCI was administered i v 15 min before NA and PBA 60 min before NA The hearts were fractionated 1 hr after the NA injection The left hand bar shows the distribution of NA uptake in control animals

Fig 7 (Right) Effect of phenoxybenzamine on the distribution of NA in heart fractions The left hand bar shows the distribution in normal hearts The other two bars show the distribution 2 hrs after 10 and 20 mg/kg PBA administered i v

Effect of phenoxybenzamine alone on the NA in the heart

Schapiro (1958) reported that PBA reduced the tissue levels of NA in adrenals spleen and hearts of rats and increased the output of catecholamines in the urine

The effect of PBA on the NA content of the different fractions of the rabbit heart was investigated in the present experiments The drug was given in a dose of either 10 mg/kg or 20 mg/kg i v to normal rabbits and 2 hrs later the hearts were removed and the NA content of each fraction was estimated

In Fig 7 it can be seen that PBA reduced the NA content of all fractions of the heart It appears that the high speed sediment may be affected to a greater extent than the other two fractions

Discussion

The finding that a single i v injection of Segontin (8 mg/kg) markedly reduces the NA content in all 3 fractions of the rabbit heart is in agreement with that of Schone and Lindner (1960) who showed that a s c injection of this drug lowered the concentration of NA in the brain and myocardium of the rat by 50 to 60 The results are also consistent with the findings that Segontin can cause a complete release of NA from the isolated adrenal medullary granules (Schone and Lindner 1962 Carlsson Hillarp and Waldeck 1963) and in higher concentrations from splenic nerve granules (Euler Stjarne and Lishajko 1964)

Rabbits treated with the above dose of Segontin developed convulsions but if a lower dose of Segontin (less than 5 mg/kg) was used it was possible to lower the tissue levels of catecholamine without producing these symptoms Therefore the fall in catecholamine levels appears to be a primary effect of the drug and not secondary to the convulsions

The uptake of NA and A by various tissues has been described by several authors including Raab 1943 Nickerson *et al* 1950 Raab and Gigee 1955 Axelrod Weil Malherbe and Tomchick 1959 Fennefather and Rand 1960 Stromblad and Nickerson 1961 Wegmann and Kako 1961 and Iversen 1963

Most of these studies were carried out by injecting or infusing catecholamines into normal animals In the present experiments the tissues were found to take up amines after the NA stores had been depleted to less than 10 % of their normal value By th present technique it was thus possible to study the net uptake of catecholamine in the tissues

An i m injection of NA restored the amine levels in each of the fractions of the heart approximately to normal The tissue levels did not remain at normal however but fell fairly rapidly after reaching a peak 2 hrs after the i m NA injection

The shortlasting repletion effect of parenteral NA administration after Segontin treatment shows that the drug had seriously impaired either the long term NA retention capacity of the tissues or the endogenous refill mechanism while not preventing temporarily accumulation of this amine in the tissues in the presence of high NA concentrations in the extracellular fluid

It is interesting to compare the rate of fall of the levels of NA in the heart after an i v infusion of the amine with the results of Kopin Hertting and Gordon (1962) who studied the release of tritiated NA from the heart of the rat after an i v infusion These authors found that in the heart of normal rats the content fell very rapidly at first then very slowly whereas in reserpine treated rats the amount of tritium labelled NA fell to zero in less than 15 min

There is much controversy in the literature about whether or not there is a difference in the uptake and release of the d and l isomers of NA and A Crout (1964) and Mueller and Shideman (1964) found no stereochemical specificity in their experiments On the other hand Kopin and Bridgers (1963) Iversen (1963) Maickel Beaven and Brodie (1963) Beaven and Maickel (1964) and Euler and Lishajko (1964) have report d that there is a difference in uptake and retention of the two isomers

The present results support the later group One criticism of this interpretation of the present findings could be that because of the difference in biological activity of the isomers at the site of injection there could be a difference in the speed of entry of th two isomers into the general circulation The present results cannot exclude this possibility However Euler and Lishajko (1964) have shown that the ATP dependent uptake in isolated splenic nerve granules of NA and A at low amine concentrations is to some extent stereo-specific It would seem therefore that any possible difference in rate of absorbtion into the circulation from the site of injection due to differences in vasoconstrictor potency between the two isomers could n t be the sole reas n for th different patterns of occurrence of the two isomers in the heart at different times

Kopin and Bridgers (1963) suggested that the uptake of the two isomers of NA was similar but that their rate of release was different The present results support th idea that the release rates are different

Brown and Gillespie (1957) reported that PBA increased the splenic venous blood concentration of NA following nerve stimulation They thought at the time that the increase in the overflow was due to the action of PBA on the receptors In 1961 Hertting Axelrod and Whitby reported that PBA decreased NA tissue levels by interfering with tissue binding in the nerve ending stores More recently Gillespie and Kirpekar (1963) and Blakeley Brown and Geffen (1964) have interpreted the action of PBA on the sympathetic transmitter in the spleen of the cat as a block of re uptake of NA

In the present experiments DCI which blocks the β receptors in the heart had but a small effect on the uptake of NA into the rabbit heart whereas PBA caused a very striking inhibition of the uptake

These findings are in agreement with Hertting Axelrod and Whitby (1961) and with the recent concepts of Brown Gillespie and coworkers

Schapiro (1958) found that PBA caused depletion of the NA in the heart of rats and increased the output in the urine The present investigations showed further that depletion occurred in all fractions of the heart and particularly in the high speed sediment In this respect it is interesting that Euler Stjarne and Lishajko (1964) found that PBA had a protective action on the isolated splenic nerve granules while it had an opposite effect on the adrenal medullary granules

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Acta physiol scand 1965 63 423—427

From the Department of Physiological Chemistry University of Lund, Sweden

The Metabolism of Fatty Acids in the Rat

IV Stearic acid

By

GORAN GORANSSON

Received 8 July 1964

Abstract

Goransson G *The metabolism of fatty acids in the rat IV Stearic acid* Acta physiol scand 1965 63 423—427 — 1 C Stearic acid and H palmitic acid in rat serum were injected i v into fasted and refed male rats and the disappearance of radioactivity from the blood, its tissue distribution and recirculation into the blood were studied Stearic acid was taken up at a lower rate than palmitic acid Five minutes after the injection about the same total amount of C as of H label was recovered from the fasted rats At 3?0 min more C label was left. This may be due to the less rapid turnover of the stearic acid-containing phospholipids compared to the glycerides In the liver there was a marked preferential incorporation of stearic acid into the phospholipids whereas the ratio of incorporated stearic acid to palmitic acid in the glycerides was low This skewed distribution was also seen in the blood phospholipids and the blood glycerides

In previous investigations of the metabolism of individual free fatty acids palmitic acid alone (Göransson and Olivecrona 1964) and in combination with oleic acid (Goransson and Olivecrona 1965) and with arachidic acid (Goransson) was injected into rats and the disappearance from the blood the tissue distribution and the recirculation into the blood were studied The present report gives the results of experiments in which palmitic acid and stearic acid were simultaneously injected into male rats

Methods

The rats the method of preparing the injection solution the operative procedures and the analyses performed on the samples have been described Goransson and Olivecrona 1964 1965

The 1-C^{14} stearic acid (batch 19, specific activity 7.46 mC/mmole) was purchased from The Radiochemical Centre, Amersham, England. It was purified twice by reversed phase chromatography using 85 % methanol/15 % water as mobile phase and heptane as stationary phase. The H^3 palmitic acid was the same preparation as used previously (Göransson). Approximately 0.2 μeq of labeled stearic acid and 0.04 μeq of labeled palmitic acid were injected in each case.

Results

The percentage of the injected dose of palmitic acid found in separate lipid fractions agreed closely with that found earlier (Göransson and Olivecrona 1964). The values in this paper are therefore given as the ratio between stearic acid label (C^{14}) and palmitic acid label (H^3), the ratio in the injected fatty acids being taken as one.

Disappearance of label from the blood (Table I)

The labeled stearic acid was extracted from the blood at a lower rate than the labeled palmitic acid. During the first 5 min in the fasted rats and the first 3 min in the refed rats the C^{14}/H^3 ratio gradually increased. At later times the ratio decreased.

Recirculation of label into the plasma (Table II)

The ratio for the blood free fatty acids continued to fall after 5 min in both the fasted and the refed rats. The ratio in the cholesterol esters in the refed rats was greater than unity throughout the time period studied.

The blood triglycerides showed a marked difference in the two nutritional states. In the fasted rats there was much less C^{14} label than H^3 label in this fraction, whereas

TABLE I. Ratio C^{14}/H^3 radioactivity in the blood free fatty acid fraction in rats after the i.v. injection of C^{14} stearic and H^3 palmitic acid in rat serum. Each figure is the mean of values from 3 rats. The ratio in the injected fatty acids was taken as 1.0.

	1	2	3	4	5	Min
Fasted	1.2	1.4	1.4	1.5	1.5	
Refed	1.4	2.2	2.5	2.1	1.6	

TABLE II. Ratio C^{14}/H^3 radioactivity in blood lipids in rats after the i.v. injection of C^{14} stearic and H^3 palmitic acid in rat serum. The ratio in the injected fatty acids was taken as 1.0.

	20	40	80	160	320	Min
Fasted						
Glycerides	0.3 0.2	0.3 0.3	0.3 0.3	0.3 0.4	0.3 0.4	
Phospholipids	0.4 0.4	0.6 0.6	0.6 0.5	0.8 0.9	0.9 1.1	
Refed						
Glycerides	0.7 0.6	1.1 1.1	1.2 0.7	0.9 1.0	0.8 1.1	
Phospholipids	0.5 0.5	0.4 —	0.8 0.8	0.9 1.0	1.1 0.8	

TABLE III Ratio C^{14}/H^3 radioactivity in tissue lipids from rats after the i.v. injection of C stearic and H palmitic acid in rat serum. The ratio in the injected fatty acids was taken ■ 1.0

Nutritional state	Minutes	Liver Total	Liver NF	Liver PLFA	Adipose tissue Total	Muscle tissue	Heart	Kidneys	Lungs	Spleen	Carcass	In the rat
Fasted	5	0.87	0.23	1.19	1.00	1.36	1.79	0.84	1.00	1.26	1.12	1.00
		0.80	0.24	1.24	1.07	1.26	1.31	0.80	1.17	1.25	1.09	1.00
Refed	5	0.89	0.72	0.96	0.90	0.83	0.65	0.[illegible]4	0.60	0.87	0.99	0.94
		0.92	0.71	0.97	0.80	—	0.73	0.86	0.61	0.90	0.90	0.89
Fasted	320	1.60	0.59	2.38	1.00	—	2.40	0.94	0.78	0.89	1.17	1.27
		1.62	0.31	2.10	1.11	1.00	2.26	0.99	0.80	0.93	1.07	1.23

in the refed rats the ratio was close to unity. No significant variation in the ratio with time was noted. The blood phospholipids had a ratio C^{14}/H^3 of about 0.5 at 20 min and a ratio close to unity at 320 min in both nutritional states.

Tissue radioactivity (Table III)

In the fasted rats equal amounts of the 2 isotopes were found after 5 min but at 320 min more C^{14} label was recovered. In the refed rats the C^{14}/H^3 ratio was slightly less than unity at 5 min.

In the livers of the fasted rats less C^{14} — than H^3 label was found at 5 min and more at 320 min. In the neutral lipids the ratio was considerably less than unity and in the phospholipids greater. This difference was much less marked in the refed rats, where both in the neutral lipids and in the phospholipids the ratio was closer to unity.

In the muscle, heart and spleen in the fasted rats the ratio of C^{14} label to H^3 label was greater than unity, in the adipose tissue and the lungs close to unity and in the kidneys smaller than unity at 5 min. At 320 min the ratio had become greater in the heart and the kidneys and smaller in the muscle tissue, the lungs and the spleen. It remained constant in the adipose tissue. The total radioactivity in the fat pad removed at 320 min was hardly greater than the background and therefore the values are of doubtful significance.

In the refed rats there was less C^{14} label than H^3 label in all tissues at 5 min.

Discussion

The disappearance of injected labeled fatty acids from the blood has been investigated by several authors (Bierman, Schwartz and Dole 1957; Fredrickson and Gordon 1958; Laurell 1957; Goransson and Olivecrona 1964, 1965; Goransson) but there is no

general agreement regarding possible differences in extraction rates of the individual fatty acids In the present work it was found that palmitic acid is more rapidly taken up by the cells than stearic acid

After the entry into the cells the fatty acids are metabolized via different pathways (Goransson and Olivecrona 1964) The most important of the latter are conversion to other fatty acids incorporation into glycerides and phospholipids with storage or recirculation back into the plasma and oxidation

Conversion to other fatty acids

Conversion of stearic acid to oleic acid has been shown to take place in refed rats In the neutral fat of the liver almost all the C^{14} injected as stearic acid is found associated with oleic acid This does not occur in fasted rats (Elovson)

Incorporation into triglycerides and phospholipids

The fatty acid composition of several lipid fractions was studied by Goransson and Olivecrona 1964) The neutral fat contained much less stearic acid than did the phospholipids In the present experiments more stearic acid label was therefore expected in the phospholipids than in the glycerides in the liver Table III shows that the ratio C^{14}/H^3 was indeed much lower in the neutral fat than in the phospholipids both at 5 and at 320 min in the fasted rats This is in agreement with the findings of Trout and Estes (1962) When the amounts of label are compared with the total amounts of the respective fatty acids found by Goransson and Olivecrona (1964) it follows that the stearic acid and the palmitic acid are incorporated in direct proportion to the fatty acid composition in the respective lipid fraction

The much smaller difference in the refed rats is explained by the extensive conversion of stearic acid to oleic acid and by the interconversion of palmitic acid found by Elovson

Recirculation of label back to the blood

Blood triglycerides and phospholipids have been shown to originate in the liver (Fishler et al 1943 Laurell 1959) One should therefore expect the blood glycerides in the present work to have about the same ratio of C^{14} label to H^3 label as the liver neutral fat This was also the case both in the fasted and in the refed rats which indicates that in the refed rats the desaturated stearic acid is recirculated into the blood in the glyceride fraction

The total radioactivity in the blood phospholipids was low until 160 and 320 min after the injection At these times the ratio between stearic acid and palmitic acid label was about the same as the ratio which was present in the liver phospholipids at 5 min It is possible that the turnover of the phospholipids is so slow that the pattern of label in the liver does not affect that in the blood until several hours after the injection

Oxidation

Palmitic acid was shown by Goransson and Olivecrona (1964) to be oxidized rapidly during the first few minutes after the injection This suggests that the fatty acid is available for oxidation immediately after its entry into the cell From 5 to 320 min the oxidation rate of palmitic acid was lower

In this work the stearic acid was oxidized at the same rate as the palmitic acid during the first 5 min

Less stearic acid label was recovered in both livers and carcass in the fasted rats than in the refed rats This is not in agreement with the findings of Trout and Estes (1962) who reported that no oxidation takes place in the livers of fasted rats during the first few minutes after injection.

At 370 min less palmitic acid label than stearic acid label was recovered This may be explained by the low turnover rate of the phospholipids (Dittmer and Hanahan 1959 Goransson and Olivecrona 1964) and the unequal distribution of the labeled stearic and palmitic acid between glycerides and phospholipids

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The Metabolism of Fatty Acids in the Rat

V Palmitoleic Acid

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Abstract

Goransson G *The metabolism of fatty acids in the rat V Palmitoleic acid* Acta physiol scand 1965 63 428—433 — H^3 palmitoleic and C^{14} palmitic acid were simultaneously injected into fasted or refed male rats and the disappearance of label from the blood the tissue distribution of label after 2 and 320 min as well as the recirculation of label into the blood were studied It was found that the injected palmitoleic acid was taken up and oxidized at a higher rate by the cells than was the injected palmitic acid At short times more H^3 label than C^{14} label was found in the neutral fat in most tissues indicating that palmitoleic acid is preferentially incorporated into the glycerides The total lipids of the heart contained more palmitoleic acid label than palmitic acid label in the refed rats In all the mentioned respects palmitoleic acid was metabolized in the same way as oleic acid

A useful method for biosynthesis of labeled palmitoleic acid from labeled palmitic acid is reported

In previous papers the results of experiments with palmitic acid alone or in combination with oleic acid arachidic acid or stearic acid have been reported (Goransson and Olivecrona 1964 1965 Goransson 1965 a b) In the present experiments H^3 labeled palmitoleic acid was injected together with C^{14} palmitic acid and the following parameters were studied 1 The disappearance of label from the circulating blood 2 The tissue distribution 3 The recirculation of label back into the blood The values for palmitoleic acid were similar to those obtained for oleic acid (Goransson and Olivecrona 1965)

Methods

In the present experiments fasted and refed male rats were iv injected with H^3 palmitoleic acid together with C^{14} palmitic acid After 2 or 320 min the rats were sacrificed and the distribution of the label determined Blood curves were obtained from separate rats at short times (1—5 min) by withdrawing small blood samples from the jugular vein of three rats of each nutritional state and at longer times by sacrificing two rats at each time interval by exsanguination.

The rats, the operative procedures and the analyses performed on the samples have been described in detail by Goransson and Olivecrona (1964 1965)

The 1 C palmitic acid (Batch 2? specific activity 3 75 mC/mmole) was purchased from The Radiochemical Centre Amersham England It was purified by reversed phase chromatography and by thin layer chromatography

The following procedure for the biosynthesis of palmitoleic acid (devised by J Elovson) was used 10 g of glucose 5 g of peptone (Bactopeptone Difco Laboratories Detroit Michigan USA) and 5 g of yeast extract (Yeast extract Difco Laboratories Detroit, Michigan USA) were dissolved in 1 litre of distilled water After sterilization of the solution 100 mg of commercial baker s yeast was added and the flask shaken for 1 hr at room temperature After this time interval 12 mg of KCN was added to the mixture (2 × 10 M) to inhibit oxidation of palmitic acid in the yeast cells The flask was shaken for another hour and then 16 mg of unlabeled and some 9-10-H labeled (20 x 10⁸ CPM) palmitate was added in the form of methyl ester dissolved in water by means of Cutscum® (Fisher Scientific Company USA) as it is known that esters enter yeast cells more readily than free fatty acids (Oura and Suomalainen 1963) After III hrs of continued shaking the yeast was recovered by centrifugation and hydrolyzed with equal volumes of concentrated HCl and water for one hour on a boiling waterbath The lipid material was extracted with petroleum ether from a 50 ethanol water solution and the fatty acids methylated with diazo-methane. The fatty acid methyl esters were separated from sterols and other more polar lipids by elution from a SiO (Mallinckrodt) column with 2 % of ether in petroleum ether and separated according to number of double bonds using an AgNO SiO column After saponification the palmitoleic acid was then isolated by reversed phase chromatography

It was found that about 80 % of the label added to the Erlenmeyer flask was taken up by the yeast cells 75 % of the label taken up was in the monounsaturated fraction and 80 % of this fraction proved to be palmitoleic acid The overall yield of palmitoleic acid was 40—50 % of the added radioactivity

Using this procedure palmitoleic acid was biosynthesized from 25 mC of 9 10-H palmitic acid obtained from The Radiochemical Centre, Amersham England. The palmitoleic acid was purified by reversed phase chromatography twice and by thin layer chromatography The specific activity of the biosynthesized acid was 7 mC/mmole

An injection solution containing approximately 0 3 μeq of labeled palmitoleic acid and 0 ? μeq of labeled palmitic acid was prepared as described by Goransson and Olivecrona (1964)

It was necessary to use H palmitic acid as a starting material in the biosynthesis of the palmitoleic acid because of the low specific activity of the commercially available C labeled palmitic acid

In the present work C palmitic acid therefore had to be used as a reference instead of H palmitic acid as in earlier experiments To confirm the similar behaviour of C and H palmitic acid these were simultaneously injected into rats No significant differences were found either in the tissue distribution or during the analyses

Results

The percentages of the injected palmitic acid radioactivity recovered in different lipid fractions were similar to those found earlier by Goransson and Olivecrona (1964) Therefore the present results are given as the ratio between palmitoleic acid label H³) and palmitic acid label (C¹⁴ The ratio in the injected fatty acids was taken as

TABLE I Ratio H C radioactivity in the blood free fatty acid fraction in rats after the injection of C palmitic and H palmitoleic acid in rat serum Each figure is the mean of values from 3 rats The ratio in the injected fatty acids was taken as 1 0

	1	2	3	4	5	Min
Fasted	0 8	0 ?	0 6?	0 ?	0 80	
Refed	0 59				0 4	

one Thus a ratio greater than unity in a lipid fraction means that a higher percentage of the injected dose of palmitoleic acid than of palmitic acid was found in that fraction

Disappearance of label from the blood (Table I) Palmitoleic acid was extracted at a higher rate by the cells than palmitic acid as can be seen from the ratio in Table I Both in the fasted and the refed rats the ratio H^3/C^{14} in blood was smaller than unity 1 min after the injection It decreased continually until 3 min after the injection in the

TABLE II Ratio H^3/C^{14} radioactivity in the blood lipids in fasted rats after the i v injection of C^{14} palmitic and H^3 palmitoleic acid in rat serum The ratio in the injected fatty acids was taken as 1 0

Min	Cholesterol esters	Free fatty acids	Glycerides	Phospholipids
10	0.3	0.8	0.6	0.8
	0.3	0.7	0.7	—
20	0.7	0.7	0.7	0.8
	0.5	0.6	0.6	0.7
40	1.8	0.7	0.6	0.5
	1.5	0.6	0.6	0.6
80	4.0	—	1.0	0.5
	—	0.6	—	0.3
160	[illegible]6	0.6	0.5	0.4
	6.3	—	0.4	0.5
320	2.3	0.6	0.5	0.4
	1.1	0.5	0.2	0.2

TABLE III Ratio H^3/C^{14} radioactivity in tissue lipids in rats after the i v injection of C ± S E M The ratio in the injected fatty acids was taken as 1 0

Min	Liver	Adipose tissue	Muscle	Heart
	Total			
	Fasted			
2	0.43 ± 0.04	1.09 ± 0.11	0.91 ± 0.06	0.[illegible] ± 0.03
320	0.21 ± 0.03	0.93 ± 0.07	0.69 ± 0.02	0.[illegible] ± 0.10
	Refed			
2	1.1[illegible] ± 0.04	1.44 ± 0.05	1.27 ± 0.05	1.4[illegible] ± 0.07
320	0.93 ± 0.01	1.40 ± 0.10	1.13 ± 0.0	0.[illegible]8 ± 0.03

fasted rats and in the refed animals the ratio at 5 min had a lower value than that at 1 min

Recirculation of label back into the blood in fasted rats (*Table II*) The H^3/C^{14} ratio in the cholesterol esters was smaller than unity at 10 min but it gradually became greater and from 80 to 320 min the ratio was considerably greater than unity However, the blood cholesterol esters showed low levels of radioactivity throughout the time period studied Thus the ratios for the cholesterol esters are somewhat uncertain In the free fatty acids there was less palmitoleic acid label than palmitic acid label throughout the experiment In the glycerides there was less palmitoleic acid label During the peak of radioactivity in the blood at 20 and 80 min the ratio was 0 6—0 7 The phospholipid H^3/C^{14} ratio declined while the total radioactivity in this fraction rose throughout the time studied At 320 min the ratio was 0 3

Tissue distribution (*Table III*) In the total body lipids of the fasted rats the ratio of palmitoleic acid label to palmitic acid label was 0 8 at 2 min and 0 45 at 320 min In the refed rats more palmitoleic acid label than palmitic acid label was found at 2 min in the liver the heart the adipose tissue and the muscle tissue In the kidneys the lungs and the spleen the ratio was close to unity At 320 min the ratio had decreased in all the organs except in the adipose tissue and the lungs In the fasted rats the adipose tissue and the muscle tissue showed a ratio close to unity at 2 min while in the other organs the ratios were smaller than unity At 320 min the ratio in all the organs had decreased below the value found at 2 min

There was consistently less palmitoleic acid label than palmitic acid label in all the lipid fractions from fasted rats than in those from refed rats

Distribution between neutral fat and phospholipids in the tissues (*Table IV*) Both at 2 and 320 min the ratio H^3/C^{14} was greater in the neutral fat than in the phospholipids in the fasted rats (except in the spleen) In all fractions studied the ratio was smaller than unity except in the muscle at 2 min

In the refed rats the pattern was more complex At 2 min the ratio was sometimes greater (liver heart and spleen) sometimes equal (muscle) and sometimes lower (kidneys and lungs) in the neutral fat than in the phospholipids At 320 min the ratio

palm tic and H palm tole c ac d in rat serum Each value is the mean of alues f om 3 rats

Kidneys	Lungs	Spleen	Carcass	In the rat
Total				
0 6? ± 0 03	0 64 ± 0 01	0 6 ± 0 02	0 91 ± 0 04	0 77 ± 0 0?
0 3? ± 0 03	0 4 ± 0 03	0 31 ± 0 0	0 58 ± 0 07	0 45 ± 0 0
1 08 ± 0 03	0 9 ± 0 0	1 0 ± 0 0	1 10 ± 0 03	1 11 ± 0 0
0 73 ± 0 07	1 10 ± 0 0?	0 60 ± 0 04	1 00 ± 0 06	0 93 ± 0 04

TABLE IV Ratio H^3/C^{14} radioactivity in the neutral lipid and the phospholipid fractions of tissue lipids in rats after the i v injection of C^{14} palmitic and H^3 palmitoleic acid in rat serum Each figure is the mean of values from 3 rats ± S E M The ratio in the injected fatty acids was taken as 1 0

Min	Lipid fraction	Liver	Muscle	Heart	Kidneys	Lungs	Spleen
	Fasted						
2	Neutral lipids	[illegible] 0±0 06	1 00±0 04	0 70±0 01	0 81±0 01	0 57±0 01	0 61±0 03
	Phospho lipids	0 07±0 01	0 93±0 0[illegible]	[illegible]31±0 0[illegible]	0 63±0 02	[illegible]±0 07	0 67±0 01
320	Neutral lipids	0 31 ±0 06	0 79±0 01	0 35±0 0	0 47±0 01	0 4±0 03	0 45±0 01
	Phospho lipid	0 04±0	0 57±0 02	0 27±0 04	0 37±0 04	0 31±0 03	0 41±0
	Refed						
2	*Neutral lipids*	*1 46±0 14*	*1 14±0 03*	*1 42±0 03*	*1 07±0 09*	*0 61±0 03*	*1 38±0 14*
	Phospho lipids	0 19±0 01	1 14±0 07	0 67±0 19	1 15±0 08	1 09±0 04	0 90±0 05
320	Neutral lipids	*1 32±0 02*	*1 00±0*	*0 60±0 03*	*0 98±0 02*	*0 96±0 07*	*0 8[illegible]±0 09*
	Phospho lipids	0 12±0 01	1 22±0 10	0 60±0 03	0 91±0 03	0 88±0 03	0 74±0 02

was lower in the phospholipids than in the neutral lipids (except in the muscle) The ratios were always lower in the fasted rats than in the refed rats and lower at 320 min than at 2 min (except in the muscle of the refed rats)

Discussion

Disappearance of label from the blood The possibility of differences in disappearance rate for separate fatty acids has been discussed by Goransson and Olivecrona (1965) Fredrickson and Gordon (1958) did not find any differences in extraction rate between palmitic oleic and linoleic acid In contrast to these findings experiments in our laboratory have given results indicating differences in the extraction rates for palmitic oleic arachidic and stearic acids (Goransson and Olivecrona 1965 Göransson 1965 a b) The present results moreover indicate that palmitoleic and palmitic acid are removed from blood at different rates *Palmitoleic acid was extracted at a considerably higher rate* When the lack of agreement between our results and those of Fredrickson and Gordon (1958) is considered it should be pointed out that Fredrickson and Gordon injected fatty acids bound to human serum albumin while in our experiments whole rat serum was used for dissolving the labeled material However further investigations are needed to explain the divergent results

Oxidation From the present data it is clear that the labeled palmitoleic acid was more rapidly oxidized than the labeled palmitic acid Both the ratios in the total body lipids and the ratios in the total tissue lipids were smaller than unity in the fasted rats The fact that the ratio became smaller at 320 min than at 2 min in the fractionated tissue lipids also indicates a greater oxidation of palmitoleic acid.

Interconversion Palmitoleic acid may be elongated to cis vaccenic acid in the liver Preliminary results show that the conversion is not extensive enough to affect the interpretation of the data in this paper (Elovson to be published) The interconversion of palmitic acid has been discussed earlier (Goransson and Olivecrona 1964)

Incorporation into glycerol esters Palmitoleic acid was rapidly incorporated into the glycerol esters of all tissues studied In the heart the ratio of palmitoleic acid label to palmitic acid label was very high in the refed rats This is in agreement with the marked arteriovenous difference for palmitoleic acid across the heart observed by Carlsten *et al* (1963) With respect to the high incorporation of palmitoleic acid into adipose and muscle tissue the high content of glycerides in these organs should be noticed (Goransson and Olivecrona (1964)

Fractionation of the total lipid extracts into neutral lipids and phospholipids gave results that showed a preferential incorporation of palmitoleic acid into the neutral lipids This corresponds very well with the difference in fatty acid composition between the neutral lipids and phospholipids reported by Goransson and Olivecrona (1964)

Recirculation of label back into the blood For oleic acid and for stearic acid it has been shown that the ratio between the respective acid and palmitic acid in the liver neutral lipids closely resembles that in the blood (Göransson and Olivecrona 1965 Goransson 1965 b) In the present study the ratio of palmitoleic acid label to palmitic acid label in the blood glycerides was also similar to that in the liver neutral lipids The turnover of the liver phospholipids was so slow that the pattern of label in the liver phospholipids did not affect that in the blood until several hours after the injection of the labeled material

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From King Gustaf V Research Institute the Department of Internal Medicine Karolinska Hospital and the Department of Medicine Royal Veterinary College Stockholm Sweden

Concentration and Turnover of the Free Fatty Acids of Plasma and Concentration of Blood Glucose during Exercise in Horses

By

LARS A CARLSON SVEN FROBERG and SUNE PERSSON

Received 16 July 1964

Abstract

Carlson L A S Froberg and S Persson *Concentration and turnover of the free fatty acids of plasma and concentration of blood glucose during exercise in horses* Acta physiol scand 1965 63 434—441 — The concentration of FFA and glycerol in plasma and of glucose and lactate in blood was studied in 5 horses during exercise The turnover of FFA was also determined During exercise the efflux of FFA from plasma as well as the mobilization of FFA into plasma was increased The glycerol concentration generally increased while the concentration of glucose fell This decrease of glucose was very pronounced in 2 of the horses The lactate concentration showed no consistent changes When epinephrine was infused into 2 horses the concentration and the turnover of FFA as well as the concentration of glycerol increased The concentration of glucose fell slightly while the lactate concentration increased significantly in response to adrenaline

During exercise in man the concentration of the free fatty acids (FFA) of plasma decreases during the first 10—15 min (Carlson and Pernow 1959 Friedberg *et al* 1960 Bruce Cobb and Williams 1961 Carlson and Pernow 1961 Konttinen *et al* 1962 Friedberg *et al* 1963 Havel Naimark and Borchgrevink 1963) in order to increase if the exercise is continued (Basu Passmore and Strong 1960 Carlson and Pernow 1961 Friedberg *et al* 1963 Havel *et al* 1963)

It has been shown with labelled fatty acids that the initial decrease of FFA during exercise is due to increased efflux of FFA from plasma (Friedberg *et al* 1960 Carlson and Pernow 1961 Friedberg *et al* 1963 Havel *et al* 1963) The increase occurring when the exercise is prolonged is due to increased mobilization of FFA (Friedberg *et al* 1963 Havel *et al* 1963) — presumably from adipose tissue The importance of FFA for the energy metabolism in the resting state has recently been reviewed and stressed (Fredrickson and Gordon 1958 Fritz 1961) Furthermore studies during exercise in the fasting state has shown that when well trained subjects walked (Havel

et al. 1963) and untrained persons bicycled (Carlson *et al.* 1963) 50 and 25 per cent, respectively, of the carbon dioxide expired during the exercise was derived from immediate oxidation of plasma FFA.

Comparatively few studies have been done on the FFA metabolism during exercise in animals. As in man the concentration of FFA in plasma decreases during exercise in dogs (Issekutz and Miller 1962, Miller, Issekutz and Rodahl 1963). However, this decrease was found to be due not only to an increased efflux of FFA from plasma but also to a decreased mobilization of FFA into the blood stream (Miller *et al.* 1963) contrary to the findings in man. No studies are available on the metabolism of plasma FFA during exercise in horses, nor is it known to what extent lipids and carbohydrates are metabolized in this species during exercise. In this study data are reported on the concentration and turnover of FFA in plasma and on the concentration of blood glucose during exercise and during infusions of adrenaline.

Methods

Animals and procedure

Horses without signs of any diseases, trained to run on a horizontal treadmill, were used. They had all free access to food and water the day before the experiment. The treadmill was driven by an electric motor at a constant speed (Lannek and Persson, to be publ.). A special harness was used to prevent accidents in case of slipping. Two mares (Horse 1 and 2), standard breed from the Solvalla trotting rink, were studied with three successive periods of exercise 2 min apart at speeds of 1.5, 4 and 5 m/sec, respectively.

The isotopic studies were performed on one stallion (Horse 3), one former military mount mare (Horse 4) and one former race horse, a gelding (Horse 5). These horses were all standard breed. Their physical activity had been minimal during the year before the study. During the exercise period each horse was running at a constant speed of 3.5 m/sec. The exercise was interrupted when the horse began to show signs of unwillingness to continue. At that time they all were sweating markedly. Two other horses, one gelding (Horse 6) and one mare (Horse 7), were given constant infusions of epinephrine. During these infusions the horses had the following symptoms: Increasing excitement, muscular tremor, profuse sweating, elevated skin temperature, T wave changes and partial A-V block. The infusions were interrupted on account of extreme excitement. Catheters were inserted percutaneously under local anesthesia (Persson, to be publ.). For blood sampling a polyethene heart catheter was inserted on the left side of the neck into the jugular vein. The pressure was recorded through the heart catheter with an Elema-Schonander pressure transducer. The position of the tip of the catheter was ascertained from the pressure curves. In one of the experiments the tip was situated in the right ventricle, in all others in the pulmonary artery. The isotope infusion was given through a teflon catheter into the right jugular vein. The tip of this catheter was placed about 3 dm caudal of the mandibular angle. In the experiments where epinephrine was infused, the drug was administered through a separate teflon catheter on the same side as the isotope. Care was taken not to introduce any heparin into the blood stream, since preliminary studies showed that i.v. injection of heparin (0.5 mg/kg) released lipoprotein lipase activity into the blood stream. The heart catheter was kept patent by a slow drip of saline. Blood was withdrawn through the heart catheter into silicone treated, heparinized syringes. The blood was immediately precipitated for the determinations of glucose and lactate. The remaining blood was kept for about 1 hr on ice before the plasma was centrifuged and processed. No hemolysis was observed.

Preparation of labelled palmitate for infusion

500 ml of blood was taken from the horse by venipuncture the day before the experiment and collected into 100 ml of ACD-solution (citric acid 0.48 g, sodium citrate 1.32 g and glucose 1.47 g per 100 ml). Plasma was prepared under sterile precautions. About 1 mC of palmitic acid-9, 10-³H (New England Nuclear Corporation, 100 mC/mmole) was neutralized with 0.0 N NaOH in slight excess, the salt warmed to about 5 C and 20 ml of the plasma added. The albumin solution was diluted to about 170 ml with the rest of plasma. This solution was infused at a rate of 1.75 ml/min during the experiment.

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Preparation [illegible]

500 ml of blood [illegible] was taken from [illegible] horse by venpuncture the day before [illegible] collected [illegible] ACD solution (citric acid 0.48 g, sodium citrate [illegible] 1.47 g per 100 [illegible]). [illegible] was prepared under sterile precautions. Albumin [illegible] 19 [illegible] Corporation) 100 mc/[illegible] 0.9 [illegible] salt warmed to about 3[illegible] C and 20 [illegible]. [illegible] about 10 ml with inactive plasma [illegible] the experiment.

Analytical methods

Plasma FFA was titrated according to Dole (1956) as modified by Trout Estes and Friedberg (1960) in duplicate Glucose was determined in duplicate with glucose oxidase according to Marks (1959) Glycerol was determined enzymatically according to Wieland (1957) The enzymatic method of Scholz *et al* (1959) was used for lactate estimations

Radioactivity in the FFA fraction was determined by taking off 3 ml of the sulfuric acid washed heptane phase obtained in the extraction procedure Inactive palmitic acid and tripalmitin — 100 µg each — was added the sample evaporated to dryness and dissolved in 200 µl of chloroform Of this solution 100 µl was spotted on thin layer plates spread with silica gel G (E Merck Darmstadt) The plates were run with 1 per cent acetic acid and 20 per cent diethyl ether in light petroleum ether The spots corresponding to the FFA fraction were quantitatively scraped off into counting vials and 1 ml of methanol added Then 10 ml of toluene containing 0 6 per cent 2 5-diphenyloxazole and 0 03 per cent 1 4 bis 2 (4 methyl 5 phenyl oxazolyl) benzene was added The samples were counted in a Tri carb liquid scintillation spectrometer (Packard Instrument Co Inc Illinois) All samples were corrected for quenching by the use of an internal standard The recovery of labelled palmitic acid with this procedure is better than 95 per cent

Calculations

The amount of infused labelled palmitate has been corrected to a value of 10 cpm/min in all cases The turnover rate of FFA was calculated according to Armstrong *et al* (1961)

Results

Exercise at increasing work loads

The results from one of the studies is given in Fig 1 The results were similar in both studies During each 5 minute period of exercise the FFA concentration decreased The FFA concentration increased after all periods of exercise The concentration of glycerol was doubled during exercise The glucose level decreased slightly

Exercise at constant work load

The results from the studies in horses 3 4 and 5 are given in Fig 2 3 and 4 respectively During the exercise the concentration of FFA decreased slightly in horses 3 and 4 and remained unchanged in horse 5 After the exercise the FFA concentration increased in the 2 first horses The radioactivity in the plasma FFA fraction decreased with about

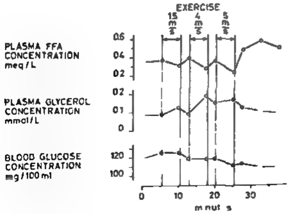


Fig 1 Concentration of FFA and glycerol in plasma and of glucose in blood before during and after exercise for 5 min at 1.5 4 and 5 m/sec in Horse 2

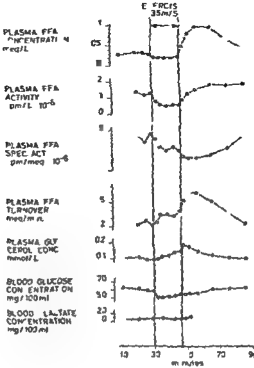


FIG 9 Concentration of FFA and glycerol in plasma and of glucose and lactate in blood before during and after exercise at 3.5 m sec in Horse 3 A continuous ■ infusion of palmitate 9 10 H was started at 0 min and continued throughout the experiment The radioactivity in the isolated plasma FFA fraction the specific activity of plasma FFA and the turnover of plasma FFA a ■ also given in the figure

50 per cent in all cases during exercise indicating increased efflux of FFA from plasma The specific activity of plasma FFA also decreased during exercise in the 3 horses This indicates increased mobilization of FFA into the blood stream The turnover of FFA at rest varied between 1—2 meq per minute During the exercise this turnover increased to about 4 meq per minute in horses 3 and 4 and to about 1 5 meq from 1 ■ meq in horse 5 The turnover of FFA increased after the exercise in horse 3 but decreased in horse 4 and 5

The concentration of glucose in blood decreased slightly during the exercise in horse 3 and decreased pronouncedly in horses 4 and 5 The concentration of lactate in blood showed minor inconsistent changes

Infusion of epinephrine

The concentration of FFA in plasma increased during and after the infusions of epinephrine (Fig 5 and 6 This increase was due to increased influx of FFA into plasma since the radioactivity in the FFA fraction remained constant while the specific activity decreased The turnover of FFA in plasma increased from about 1 meq per minute at rest to 8 and 4 meq respectively in the two studies The level of glycerol in plasma increased in both horses during the infusions of epinephrine

The concentration of glucose in blood decreased about 15 mg per 100 ml during the first minutes of the fusion and then slowly increased Lactate was followed in horse 7 (Fig ■) and the concentration was found to increase 5–6 times

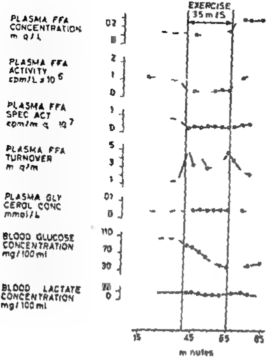


Fig 3 Concentration of FFA and glycerol in plasma and of glucose and lactate in blood before during and after exercise at 3.5 m/sec in Horse 4 A continuous i.v infusion of palmitate 9 10 H was started at 0 min and continued throughout the experiment The radioactivity in the isolated plasma FFA fraction the specific activity of plasma FFA and the turnover of plasma FFA are also given in the figure

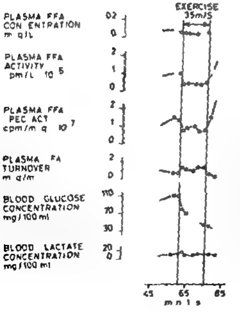
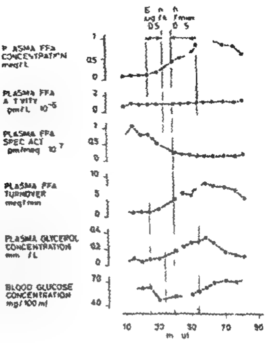


Fig 4 Concentration of FFA in plasma and of glucose and lactate in blood before during and after exercise at 3.5 m/sec in Horse 5 A continuous i.v infusion of palmitate 9 10 H was started at 0 min and continued throughout the experiment The radioactivity in the isolated plasma FFA fraction the specific activity of plasma FFA and the turnover of plasma FFA are also given in the figure

Fig 5 Concentrat on of FFA and glycerol in plasma and of glucose in blood before during and after 2 i v infus ons of ep nephrine into Horse 6 A continuous i v infusion of palmitate 9 10 H was started at 0 min and continued throughout the experiment The rad oactivity in the i olated plasma FFA fraction the spec fic activity of plasma FFA and the turnover of plasma FFA are also give i in the figure



Discussion

The two main findings of this study are The efflux from as well as the mobilization into the blood of FFA increased and the concentration of glucose in blood decreased when the horses exercised These findings could be explained if FFA as well as glucose were used in increased amounts by the exercising muscles This suggestion is supported by the studies of Zuntz and Hagemann (1898) They found that the respiratory quotient of horses decreased from a value of around 0 95 at rest to about 0 80 during exercise Increased efflux of FFA from the blood stream during exercise has been observed in man (Friedberg *et al* 1960 Carlson and Pernow 1961 Friedberg *et al* 1963 Havel *et al* 1963) and in dogs (Miller *et al* 1963) The mechanism for this increased efflux is unknown, but it has been suggested that the increased blood flow during exercise is an important factor in this context (Carlson and Pernow 1961)

In man exercise has been shown to increase the rate of mobilization of FFA into the blood stream (Friedberg *et al* 1963 Havel *et al* 1963 Carlson *et al* 1963) It is not known which factor(s) stimulate the mobilization of FFA during exercise The sympathetic nervous system may be involved in this stimulated lipid mobilization since its activity increases during exercise in man (Euler and Hellner 1955 Holmgren 1956) and catecholamines are known to increase the mobilization of FFA from adipose tissue It is of interest in this context that the horses responded to adrenaline with increased lipid mobilization The increase of glycerol in plasma during exercise has earlier been observed in man (Carlson Ekelund and Oro 1963) Havel *et al* 1963 This increase may be due to increased lipolysis in adipose tissue The plasma concentration

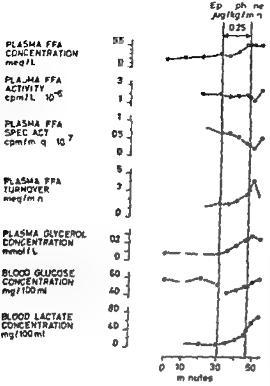


Fig. 6. Concentration of FFA and glycerol in plasma and of glucose and lactate in blood during i.v. infusion of epinephrine into Horse 7. A continuous i.v. infusion of palmitate-9,10-3H was started at 0 min and continued throughout the experiment. The radioactivity in the isolated plasma FFA fraction, the specific activity of plasma FFA and the turnover of plasma FFA are also given in the figure.

of glycerol increases after administration of norepinephrine to rabbits (Hagen 1961) and man (Carlson and Oro 1963), and, as shown here, in horses after epinephrine.

In studies on dogs the mobilization of FFA was found to be decreased during exercise (Miller *et al.* 1963). In these studies, contrary to studies in man (Havel *et al.* 1963, Carlson *et al.* 1963) and to the present studies, the concentration of glucose and lactate in blood increased significantly. The elevated levels of glucose and lactate might have depressed the mobilization of FFA. In fact, administration of glucose to exercising persons abolishes the FFA mobilizing effect of exercise (Havel *et al.* 1963, Carlson *et al.* 1963).

The decrease of the concentration of glucose in blood may be due to increased peripheral utilization or to decreased influx of glucose into the blood, or both. Earlier studies have shown that after racing in trotters the blood glucose has been found to increase (Bogdanow *et al.* 1935). At the same time the concentration of lactate in blood increased 5—10 times. Under our conditions, however, no increase in lactate was found

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Acta physiol scand 1965 63 442—449

From the Department of Physiology University of Goteborg Sweden

Supraspinal Control of the Intestino-intestinal Inhibitory Reflex

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Abstract

Johansson B O Jonsson and B Ljung *Supraspinal control of the intestino-intestinal inhibitory reflex* Acta physiol scand 1965 63 442—449 — Experiments were performed on chloralose urethane anesthetized vagotomized cats Changes in the level of adrenomedullary hormones were prevented Distension of a segment of distal jejunum produced a prompt decrease in tone and rhythmicity in the proximal jejunum This so called intestino intestinal inhibition is a propriospinal reflex response apparently mediated by sympathetic efferents Electrical stimulation in the mediocaudal part of the rhomboid fossa was found to block this spinal intestino inhibitory reflex The fact that bulbar stimulation alone sometimes produced an increase in gut motility seemed to be due to suppression of a prevailing activity in the propriospinal reflex When there was a low level of such activity medullary stimulation *per se* had little or no effect on intestinal motility It is suggested that the supraspinal structures exert an inhibitory influence on impulse transmission in the propriospinal intestino-inhibitory reflex arcs

Splanchnic afferents originating in the abdominal viscera exert a reflex influence on skeletal muscle motoneurones (e g Downman and McSwiney 1946 Euler and Sjostrand 1947) Some of these spinal reflex mechanisms have been studied with respect to the control exerted upon them from higher levels of the CNS It was found by Downman (1955) that the reflex response elicited in intercostal nerves by splanchnic stimulation was augmented after transection of the cervical spinal cord in decerebrated animals Further experiments by Downman and Hussain (1958) indicated that structures in the lower medulla exerted a tonic restraint on this splanchnic to-intercostal reflex by way of fibre systems descending in the dorsolateral fasciculi A similar organization of the control of impulse transmission from flexion reflex afferents to flexor motorneurones has been demonstrated by Holmqvist and Lundberg (1959 1961) The possibility of such supraspinal reflex control within the autonomic nervous system has apparently been investigated only with regard to the galvanic skin reflex Wang Stein and Brown (1956 a b) and Wang and Brown (1956 a b) demonstrated

in a series of papers that this propriospinal reflex is subjected to an inhibitory influence from the bulbar ventromedial reticular formation.

Different autonomic reflex responses are elicited from splanchnic afferents and at least some of these effects are mediated over propriospinal reflex connections. The intestino-intestinal inhibitory reflex, i.e. the inhibition of tone and rhythmicity produced in one segment of intestine by distension of some other separate section of the gut, represents such an autonomic spinal reflex (Chang and Hsu 1934, Johansson and Langston 1964). A similar kind of intestinal inhibition can be elicited from different intra-abdominal regions, e.g. parietal peritoneum, ureter etc. If the principle of supraspinal reflex control, discussed above with regard to somatomotor functions, operates also for autonomic splanchnic reflexes, higher centres may influence intestinal motility by modifying, in either direction, the impulse transmission in those segmental reflex arcs which subserve the intestino-intestinal inhibition. The effector result of activation or inactivation of such supraspinal control mechanisms would then be largely dependent upon the prevailing level of activity in the propriospinal reflex arc.

The aim of the present investigation was to study, in vagotomized animals, the possible influence of bulbar structures on the intestino-intestinal inhibitory response and also to find out whether the effect of bulbar stimulation on intestinal motility was related to the actual level of activity in this spinal reflex.

Methods

21 cats were used in these experiments. The animals were anesthetized by i.v. administration of chloralose and urethane (40 and 80 mg/kg respectively) after induction with ether. In all experiments a tracheal cannula was inserted. Blood pressure was recorded in one of the femoral arteries by means of a mercury manometer. Both vagi were dissected free and cut at the cervical level.

The abdomen was opened by a midline incision. Two separate segments of the jejunum with intact mesenteric nerve and blood supply were prepared by dividing the intestine between ligatures. The proximal 10 cm of the jejunum were used for registration of intestinal motility. A glass cannula was inserted into the lumen at the distal end and connected to a small reservoir. The system was filled with saline and the volume changes in the reservoir were recorded by means of a piston recorder. The pressure was kept fairly constant at 5—10 cm H_2O in this loop of intestine. The next 5—10 cm of the jejunum were connected in a similar way to a syringe filled with saline and this loop could thus be subjected to graded distensions. Intraluminal pressure was measured by means of a water manometer. The rest of the small and the whole of the large intestine were removed.

To avoid the influence of the adrenomedullary hormones on gut motility either both adrenal glands were carefully ligated and the loss of corticosteroids was compensated by intramuscular administration of hydrocortisone (5 mg/kg b.w.) or the left adrenal gland was denervated by cutting the splanchnic nerves on this side while the right adrenal was ligated. During the experiments the abdomen was either closed or kept open, but in the latter case it was covered with gauze moistened with warm saline and then with a plastic sheet to minimize cooling by evaporation.

In order to gain access to the dorsal surface of the medulla oblongata the posterior neck muscles were divided and the medial part of the occipital bone removed. The atlanto-occipital membrane and the dura were cut open and the posterior part of the cerebellum was carefully freed from the surface of the medulla and eventually sucked out. The head was fixed in a Horsley-Clarke stereotaxic instrument. The medulla was stimulated with either a concentric bipolar electrode or a pair of pointed electrodes approximately 2 mm apart. Square wave stimuli with variable impulse frequency, duration and intensity were delivered by a Grass Stimulator type S4 via a stimulus isolation unit. To avoid respiratory disturbances caused by medullary stimulation the animals were artificially ventilated with a pump set at a rate and tidal volume which barely suppressed the spontaneous breathing.

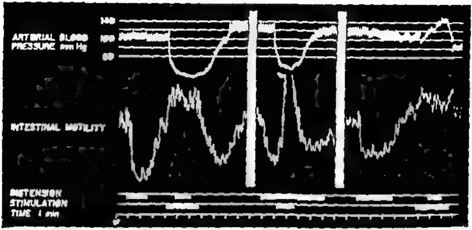

Fig 1 Cat 3 2 kg Chloralose urethane Artificially ventilated Left panel Effects of graded gut distension on intestinal motility before and during stimulation of medullary depressor area Note that no intestinal inhibition is obtained by distension during stimulation of the medulla Middle panel Effect of identical bulbar stimulation during prolonged period of distension Intestinal tone is considerably increased under these circumstances (cf effect of bulbar stimulation in the left panel) Right panel Same series of procedures as in the left panel but with stimulation of a different point in the medulla Note that stimulation here gives blood pressure rise together with elimination of the intestino-inhibitory response to distension

Results

Fig 1 illustrates a recording of arterial blood pressure and of motility in the proximal jejunum taken from an experiment where the effects of intestinal distension and bulbar stimulation were studied In the left panel of the figure distension of the distal intestinal loop to an intraluminal pressure of 100 cm H_2O is first seen to induce the characteristic inhibition of tone and rhythmicity in the proximal jejunum When the intestine had afterwards regained its previous activity a region of the medulla just lateral to the obex, was stimulated with 60 imp/sec 3 msec and 5 V This caused a marked fall of arterial blood pressure and possibly some slight increase in intestinal motility The most notable observation illustrated in this panel is however that gut distension to the same pressure as before had now no effect on intestinal motility when performed during the period of bulbar stimulation After the end of the stimulation there was a temporary decrease in intestinal tone associated with a return of blood pressure to the control level

In the second panel of Fig 1 intestinal inhibition is brought about by a prolonged distension of the distal gut segment When the same type of medullary stimulation as used in the left panel is given during the period of distension a prompt and pronounced increase in intestinal tone occurs together with a fall in blood pressure comparable to that previously obtained At the end of the bulbar stimulation intestinal tone decreased again due to the continued distension Comparing the intestinal effects of the medullary stimulation by itself in the first two panels of Fig 1 it is evident that a much greater increase in motility is elicited during the period of reflexly inhibited intestinal activity in the second panel

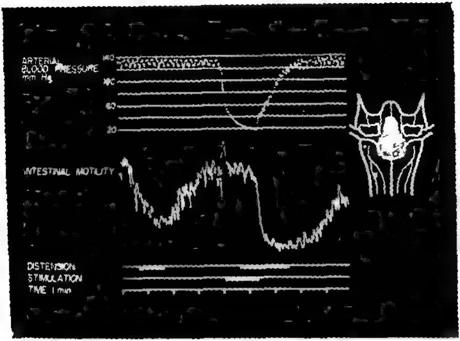

Fig 2 Cat 3 4 kg Chloralose urethane Artificially ventilated Effects of gut distension and medullary stimulation on intestinal motility Medullary stimulation which alone has no effect on intestinal activity seems to block the intestinal response to distension When bulbar stimulation is stopped there is a prompt decrease in intestinal tone due to continued distention The region stimulated is indicated by a ring in the diagram The shaded area of the diagram represents points from which intestinal motor responses and elimination of the intestino-inhibitory reflex have been produced Its dorsoventral extension seems to be considerable mainly occupying the area between 2 and 8 mm below the dorsal surface

It appears from the first panel of Fig 1 as if the intestino-intestinal inhibitory reflex was blocked in one way or another during bulbar stimulation Such an elimination of the intestino-inhibitory reflex is seen also in the third panel of Fig 1 where a point situated 2 mm dorsally to the previous one was stimulated This region produced a rise in blood pressure in contrast to the depressor effect obtained from the ventral point

In the experiment illustrated in Fig 2 there was initially a high level of spontaneous motility in the proximal segment of the jejunum A somewhat sluggish but clear-cut inhibition of tone was produced by distension of the distal loop to a pressure of 45 cm H_2O After recovery of intestinal activity a region of the medulla indicated by a ring in the inset diagram of Fig 2 was stimulated at 60 imp sec 2 msec and 4 V This stimulation produced a drastic fall in blood pressure but had by itself no significant effect on intestinal motility However exactly the same gut distension as before did not inhibit intestinal tone at all while the bulbar stimulation was continued but as soon as the latter was stopped there was a prompt and pronounced decrease in intestinal tone despite the fact that the intestinal distension was kept unchanged This abrupt intestinal relaxation preceeded the recovery of blood pressure When the distension was discontinued intestinal activity gradually recovered In this experiment also the intestinal

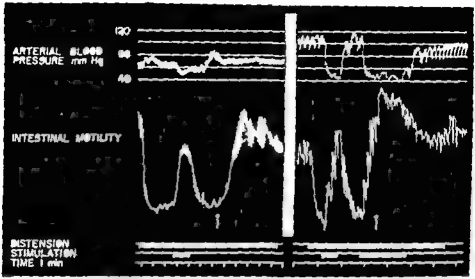

Fig 3 Left panel Cat 3 7 kg Chloralose urethane Artificially ventilated Effects on blood pressure and intestinal motility produced by medullary stimulation and extrinsic "denervation" of the gut Nerves around the superior mesenteric artery infiltrated with lidocaine at arrow Note that medullary stimulation and "denervation" produce comparable increases in motility when done during continuous distension Right panel Cat 3 5 kg Chloralose urethane Artificially ventilated Effects of medullary stimultion during prolonged gut distension Blocking of the periarterial nerves (arrow) during second period of medullary stimulation does not significantly alter intestinal activity

inhibitory response to graded gut distension was apparently blocked by stimulating medullary structures which alone did not seem to affect gut motility

Bulbar regions with a blocking influence on the intestino-intestinal reflex response could be demonstrated in all experiments and the shaded area of the diagram in Fig 2 indicates the approximate locations of the electrodes no more exact mapping has been performed so far It should be pointed out however that very strong reflex activations for instance by high distending pressures or by strong electrical stimulations of mesenteric afferents could produce intestinal inhibition even during bulbar stimulation although the reflex response was then definitely reduced When intestinal tone was initially high the medullary stimulations *per se* usually had no effect on the intestine However when intestinal tone was initially low either spontaneously or due to a maintained experimentally induced inhibitory reflex medullary stimulation often increased intestinal activity

Attempts have been made to elucidate the mechanism by which bulbar stimulation produced increased intestinal motility in these vagotomized animals Fig 3 illustrates recordings obtained in two different experiments where the intestine was finally deprived of all extrinsic nervous influence by anesthetizing the nerves around the superior mesenteric artery with 2 per cent lidocaine The local anesthetic was administered *via* a polyethylene cannula in contact with a piece of cotton covering the carefully exposed nerves In the left panel the intestino-inhibitory response to prolonged distension was first interrupted by medullary stimulation during the period indicated

leading to a raised intestinal activity A similar degree of increased intestinal activity was obtained also by anesthetizing the periarterial nerves (arrow) Comparable increases of intestinal motility were thus initiated by bulbar stimulation and by extrinsic denervation

The right panel illustrates again the effect on intestinal activity obtained by prolonged distension and by superimposed medullary stimulation which caused a return of tone and motility During a prolonged second period of identical bulbar stimulation the periarterial nerves were now anesthetized as described above This interruption of the extrinsic nervous influence on the intestine caused if anything a further increase of tone and peristalsis This fact suggests that the increased intestinal activity which occured on bulbar stimulation was brought about by a suppression of an inhibitory influence on the smooth muscles rather than by an activation of excitatory fibres to these effectors It has also been observed in this series of experiments that the intestinal response to bulbar stimulation could be abolished by intravenous injection of guanethidine (1 mg/kg) indicating that adrenergic fibres are involved Stimulation of the distal end of the cut vagal nerves at low impulse rates induced a marked increase in intestinal motility even after guanethidine

Discussion

The inhibition of motility produced in one section of intestine by distension of some other part of the gut is a spinal reflex response apparently mediated by sympathetic intestino-inhibitory efferents (Chang and Hsu 1954 Johansson and Langston 1964) The present experiments performed on vagotomized animals have shown that this effect of graded gut distensions can be blocked or greatly reduced by stimulating certain areas in the lower medulla (panel 1 and 3 in Fig 1 and Fig 2) It seems possible to explain this elimination of the reflex response by either of two mechanisms There might exist a system of fibres apart from the vagal nerves which exert a stimulating action on the smooth muscles of the intestinal wall and which when activated from the medulla make the intestine less responsive to the inhibitory influence of the intestino-intestinal reflex efferents Alternatively the bulbar stimulation might activate descending fibres which exert an inhibitory control of impulse transmission in the propriospinal reflex arcs which mediate the intestino-intestinal response

The experimental results presented in Fig 3 above seem to speak strongly in favour of the latter mechanisms Elimination of the extrinsic nervous influence on intestinal motility by blocking the periarterial nerves with a local anaesthetic produced an increase in intestinal activity of the same order of magnitude as that obtained by bulbar stimulation (first panel of Fig 3) On the other hand there was no significant change in motility when the mesenteric nerves were blocked during a period of high intestinal activity *initiated by stimulation of the medulla* (second panel of Fig 3) This indicates that bulbar stimulation produced the intestinal motor response in these vagotomized animals by releasing the smooth muscles from an inhibitory influence rather then by activating intestino-excitatory nerve fibres Furthermore the observation that guanethidine abolished the intestinal motor response to bulbar stimulation without affecting the response to efferent vagal nerve stimulation suggests that the medullary structures operate by changing the impulse activity in adrenergic nerve fibres It seems therefore as if the supraspinal region exerted its intestinal action by suppressing the impulse tran mission in the propriospinal intestino-inhibitory reflex arc The fact that bulbar

stimulation produced the most clear-cut increase in intestinal tone when performed during a period of strong reflex inhibitory influence on the intestine (cf first and second panel of Fig 1) is in conformity with this conclusion

A recent investigation by Semba Noda and Fujii (1963) concerning the effects of medullary stimulation on gastric motility in vagotomized dogs is of great interest in the light of the present findings Semba and co-workers found that movements of the stomach could be elicited from the region of the ala cinerea and from the lateral aspects of the dorsal horns in the cervical and thoracic spinal cord The responses were abolished after transection of the thoracolumbar dorsal roots They suggested that the effects of bulbar stimulation were due to activation of efferent nerve fibres which reached the splanchnic nerves *via* the dorsal roots and exerted an excitatory action on gastric motility It is possible however that the increased gastric motility could have been produced instead by elimination of a tonic propriospinal inhibitory influence by analogy with the mechanism suggested for the intestinal responses in the present study There is probably always some tonic activity in the inhibitory sympathetic efferents when the abdomen has been opened The effect of the dorsal root transection on the gastric responses is compatible with this latter explanation since this procedure would interrupt on the afferent side the spinal reflex arc which according to this view is the "substrate" of the medullary control mechanism Direct stimulation of the peripheral end of cut thoraco-lumbar dorsal roots had no effect on gut motility in the experiments by Hukuhara (1934) but was found by Kuré Ichiko and Ishikawa (1931) and by Semba and Hiraoka (1957) to produce increased gastro-intestinal movements These latter results may not necessarily mean that the alimentary tract is innervated by dorsal root efferents since antidromic stimulation of afferent nerve fibres may possibly produce gastro-intestinal responses analogous to the effect of antidromic stimulation of thin afferent fibres on cutaneous vessels (the axon reflex mechanism) The concept of a spinal parasympathetic system of dorsal root efferents proposed by Kuré and co-workers has not been generally accepted (Kuntz 1953)

The elimination of the intestino-intestinal response during bulbar stimulation was often associated in the present experiments with a pronounced fall in blood pressure due to activation of the medullary "depressor area" (left panel of Fig 1 and Fig 2) It was however possible to "block" the intestinal reflex also from more rostral and dorsal regions of the medulla which did not produce any decrease in blood pressure (third panel of Fig 1) It seems therefore as if the structures responsible for the intestinal effects do not correspond exactly to those which inhibit cardiovascular sympathetic neurones It is also evident that the intestinal responses to medullary stimulation and the induced changes in reflex activity are not secondary to the circulatory adjustments.

Preliminary experiments have indicated that the intestino-intestinal inhibitory reflex is subjected to a tonic restraint, which can be abolished by dorsal hemisection of the cervical spinal cord (Johansson Jonsson and Ljung unpublished) A supraspinal reflex control similar to that described for somatomotor reflexes (Downman 1955 Holmqvist and Lundberg 1959 1961) and for the galvanic skin reflex (Wang Stein and Brown 1956 a,b Wang and Brown 1956 a,b) may thus be operating also on gastro-intestinal spinal reflexes Different supraspinal autonomic structures may thus influence intestinal motility in the vagotomized animal by adjusting in either direction, the impulse transmission in the propriospinal intestino-intestinal inhibitory reflex arc The gastrointestinal effects of stimulation of such higher centres must then always be judged in relation to the actual level of activity in the spinal reflex

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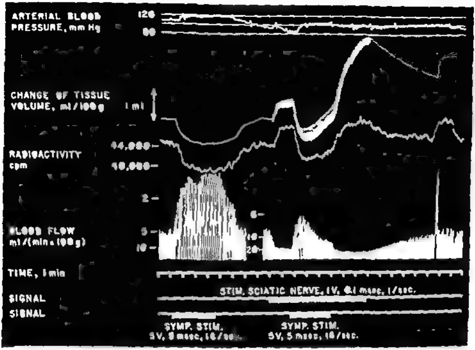


Fig 1 Cat 3 8 kg Effect of sympathetic stimulation during rest and exercise 1/sec on blood pressure tissue volume radioactivity from the calf and blood flow Note that the blood flow scale was changed immediately before the exercise After the exercise the venous outflow was abruptly raised by 15 cm H_2O for about 2 min to allow comparison between the change in tissue volume and in radioactivity The radioactivity curve was transposed photographically from the original record

tion At lower frequencies *i e* 1—2 imp/sec the vasoconstriction was only slight but persisted throughout the stimulation period At higher frequencies (generally above 4/sec) the initial vasoconstriction was marked but subsided within some minutes to persist at a plateau just above exercising level Fig 1 shows the effects of sympathetic stimulation that induced marked vasoconstriction both during rest and exercise *During* rest the vasoconstriction was well maintained throughout the stimulation period but during exercise the constrictor response ceased within a few minutes

Fig 2 A shows the frequency response curves for the blood flow resistance during simultaneous sympathetic stimulation and exercise (1 contraction per sec) in 16 expts compared with the same relationship during rest Both during rest and exercise the resistance values were noted twice for each sympathetic stimulation — first during maximum constriction and then when resistance had weakened and attained a new steady state It was not difficult to detect the latter level during exercise but during rest the resistance tended to weaken slightly throughout the stimulation period The values recorded after 8—10 min of sympathetic stimulation were therefore somewhat arbitrarily taken as steady state levels Sympathetic stimulation during rest increased the peripheral resistance up to more than fourfold and these constrictions tended to subside only slightly even during long periods of stimulation

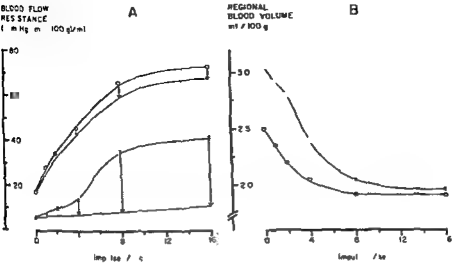


Fig 2 Frequency response curves for 16 cats 67 sympathetic stimulations during rest and 57 during exercise of one twitch sec Panel A shows the blood flow resistance panel B the regional blood volume Circles = values during rest crosses = values during exercise The flow resistance was determined during maximum constriction and during the steady state conditions. The changes from the former to the latter values are denoted by the arrows

Stimulation of the vasoconstrictor fibres during exercise of the leg produced up to a sixfold increase of the resistance to flow but within a few min the resistance fell — despite continued stimulation — to a level just above that noted during exercise only (The resistance value during exercise alone is given by the value at 0 sympathetic imp/sec)

At higher intensities of exercise the effect of sympathetic stimulation on the resistance was weaker and shorter

The vasoconstrictor response to sympathetic nerve stimulation during exercise was the same when the blood flow was kept constant by the pump as when it was allowed to vary

Capacitance

A *Tissue volume* Sympathetic stimulation induced a rapid decrease of tissue volume during rest as well as during exercise (Fig 1) This rapid decrease in volume which occurred simultaneously with the reduction of blood flow was taken as a sign of change in regional blood volume Fig 2 B gives the frequency response curves for changes of regional blood volume recorded on sympathetic stimulation in 16 cats The resting regional blood volume of 2 5 ml 100 g was taken as a reasonable estimate (cf Mellander 1960 p 38) The regional blood volume increased 0 56 ml/100 g during exercise This increase was due mainly to distension of capacitance vessels (Kjellmer 1964)

During rest the reduction of the tissue volume persisted throughout the period of sympathetic stimulation During exercise the tissue volume began to increase again simultaneously with the decreasing resistance response (Fig 1) When the period of

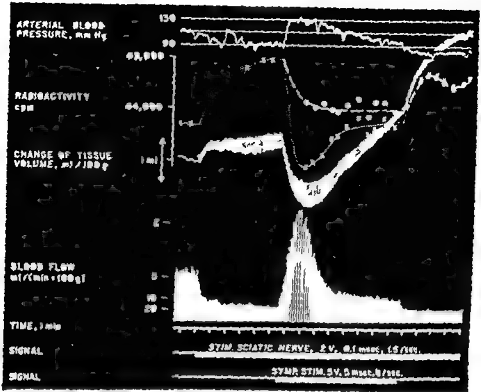


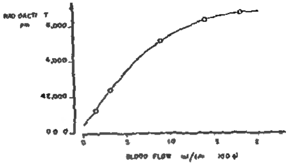
Fig 3 Cat 4 0 kg Effect of sympathetic stimulation during exercise on blood pressure tissue volume radioactivity from the calf and blood flow The radioactivity was recorded continuously with the ratemeter and counted at intervals with the scaler The scaler counts are indicated by crosses The connecting line is drawn from the ratemeter curve Using the relation in Fig 4 the passive response due to reduction of blood flow was calculated and was subtracted from the total response to give the active response (circles) The distance between total response (dotted line and the active response (dashed line) is thus the passive response calculated from Fig 4

constrictor fibre stimulation was long enough the tissue volume increased to exceed that it had before sympathetic stimulation (Fig 3) Assuming that this phase of volume gain represented the joint effect of changes in regional blood volume and in capillary filtration the radioactivity was also followed in order to estimate what proportion of the increase could be ascribed to changes in the regional blood volume

B Radioactivity The changes in the radioactivity from the calf roughly parallelled those in tissue volume Thus, the radioactivity decreased and remained low during sympathetic stimulation at rest When exercise was started the radioactivity increased simultaneously with the rapid phase of volume increase and on superimposition of vasoconstrictor fibre stimulation both the radioactivity and tissue volume decreased During the declining response of the resistance however the radioactivity returned towards control level much slower than did tissue volume — Fig 3

The decrease of regional blood volume during sympathetic stimulation depends on two factors 1 a passive component due to decreased postcapillary distending pressure

Fig 4 Same exp as in Fig 3 Relation between passive changes of radioactivity and changes of blood flow induced by the graded m chanical obstruct on of the cognate artery



caused by the reduction of blood flow and 2 an active component due to a true increase in tone of the capacitance vessels

To separate these two components of the capacitance response three different procedures were adopted 1 The capacitance response to sympathetic stimulation during exercise was studied under constant flow conditions and was found to be much better preserved than the resistance response 2 The capacitance response to sympathetic stimulation was measured at different levels of venous outflow pressure The lower the venous pressure the more marked was the decrease in volume both during rest and exercise and the higher the venous pressure during exercise the better the maintenance of the response 3 The femoral artery was gradually obstructed with a screw clamp during steady state exercise and the relationship between decrease of blood volume (in terms of decrease of radioactivity) and graded reductions of blood flow was noted It was then possible to calculate roughly what part of the total decrease of radioactivity during sympathetic stimulation was due to a passive response — a decreased distension of the capacitance vessels because of the reduced blood flow The remaining change of the radioactivity would then reflect the true constriction of the capacitance vessels Fig 4 gives the relationship between graded flow reductions and the passive decrease of radioactivity in the same experiment as that shown in Fig 3 The values in Fig 4 were obtained during the same period of exercise about ten minutes after the sympathetic stimulation had been stopped Fig 3 gives the radioactivity recorded (crosses) For each level of flow reduction the passive component was estimated from Fig 4 and subtracted from the total changes during sympathetic stimulation The results (circles) show the active response of the capacitance vessels It is obvious that while the passive component of the capacitance response weakened with the resistance response the active component persisted almost unchanged during the whole period of sympathetic stimulation

A typical finding was that as soon as the active change of tone could be evaluated separately it was found to persist unchanged or to be reduced by at most 50 per cent while the response of the resistance vessels eventually almost disappeared

CFC The capillary filtration coefficient (CFC) was determined in 7 expts during rest and exercise Vasoconstrictor fibre stimulation was then superimposed during exercise and the CFC value was re-determined as soon as the blood flow had again become steady Vasoconstrictor fibre stimulation at low frequencies — 1 to 2 imp sec — produced at most small changes of CFC, but as soon as the vasoconstriction was intense

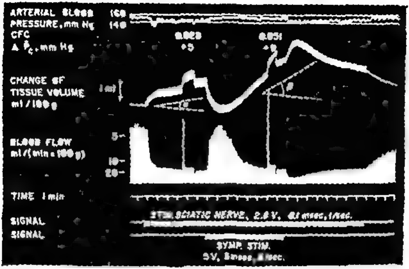


Fig 5 Cat 3 8 kg Effect of sympathetic stimulation during exercise on blood pressure capillary filtration coefficient (CFC) mean capillary pressure ($\Delta\,\bar{P}$ values denote changes relative resting level) tissue volume and blood flow CFC during rest immediately before exercise 0 014

enough substantially to impede blood flow the CFC values tended to increase further (*Fig 5*) In no instance was any reduction of the CFC observed during sympathetic stimulation During rest the mean value was 0 014 ml/(min × mm Hg 100 g) and rose to 0 022 during exercise with 1 imp/sec When a sympathetic stimulation of 1 or 2 imp/sec was superimposed no changes occurred at 4 imp/sec the CFC rose to 0 034 and at 8/sec to 0 030 (mean values

When blood flow was kept constant the CFC also rose during exercise Superimposed sympathetic stimulation then produced *no further change* in the CFC

Mean capillary pressure The slow continuous increase of volume when blood flow had reached a high constant level during steady state exercise was due to outward capillary filtration following a rise of the mean capillary pressure (Kjellmer 1964) The magnitude of this pressure rise can be calculated since both the filtration and filtration coefficients are known In these experiments exercise increased the mean capillary pressure by 5—10 mm Hg When sympathetic stimulation was superimposed it was not possible to estimate changes of capillary pressure until the blood flow had again become steady Then the sympathetic stimulation during exercise caused a further elevation of the mean capillary pressure — in the 7 expts where the CFC was determined the mean capillary pressures were increased further by 0—10 mm Hg depending on the degree of sympathetic activation Fig 5 shows how these values were obtained The angle between the control slope and the filtration slope during exercise is designated α It shows that the amount of filtrate formed per minute per 100 g was 0 14 ml Since the CFC was 0 028 ml/(min × mm Hg 100 g) the mean capillary pressure must have increased by *5 mm Hg compared with its level during rest During exercise + sympathetic activity filtration is increased — angle β which corresponds to a mean capillary pressure of* 9 mm Hg above that noted during rest

Discussion

Rein and his co-workers analyzed the effect of sympathetic activation and of adrenaline injected i v on the vascular resistance of the resting and exercising skeletal muscle (Rein 1930) Their finding that sympathetic activation caused less efficient vasoconstriction in exercising than in resting muscles has since been confirmed by *e g* Remensnyder Mitchell and Sarnoff (1962) The present results are in line with the aforementioned findings but also show that the initial response to sympathetic stimulation during exercise need not be diminished while the steady state response is very much reduced and secondly that the different consecutive vascular sections in the muscle differ from one another in functional behaviour

When the maximal constrictions obtained by sympathetic stimulation during rest and exercise were compared with prestimulatory values and expressed in terms of relative changes of blood flow resistance no difference in magnitude of the response was recorded except at the lowest frequencies of sympathetic stimulation and during the most severe muscular exercise Thus the ability of the sympathetic constrictor fibres to reduce blood flow initially may be as great in the exercising as in the resting muscle The important difference is however that the resistance response to increased sympathetic activity soon wears off in the exercising but not in the resting muscle During exercise the reduction of the vasoconstrictor response occurred after some minutes both in those experiments where blood flow was allowed to decrease and in the constant perfusion experiments This makes it less likely that the reduction of constrictor response was due primarily to an accumulation of vasodilator metabolites during sympathetic stimulation

Assuming that the capillary permeability does not change during sympathetic stimulation the changes of CFC may be regarded as manifestations of changes in the tone of the precapillary sphincters The fact that the CFC never decreased during sympathetic stimulation but tended to rise when the blood flow was restricted (Fig 5) suggests that the precapillary sphincters are particularly sensitive to the locally released metabolites — an observation in line with the findings of Cobbold *et al* (1963) in resting muscle

The capacitance vessels responded to sympathetic stimulation by expelling up to 0 6 ml blood/100 g during rest (Fig 2 B) — a figure in close agreement with the value found by Mellander (1960) During muscular activity the sympathetic stimulation induced a decrease of regional blood volume by at most 1 1 ml/100 g (Fig 2 B) which means that the extra blood accumulated in the capacitance vessels during exercise was expelled together with the amount of blood that could be mobilized already during rest Thus activation of the sympathetic vasoconstrictor fibres is a potent mechanism for an abrupt reduction of the amount of blood pooled in the muscle during exercise

To separate the active from the passive component of the capacitance response the relative magnitudes of the two components were varied By keeping the blood flow constant the passive effects were reduced (but not abolished since some changes of capillary and postcapillary pressure still occurred) By elevating the venous outflow pressure the passive response was likewise reduced since the effect of changes in distending pressure is strongest when the veins are not already fully distended (*cf* Öberg 1965) Both these procedures showed that the active constriction of the capacitance vessels was better maintained than that of the resistance vessels The same result

was obtained in the experiments where the passive component of the capacitance response was evaluated by mechanical reduction of the blood flow (Fig 3)

It may therefore be concluded that the tone of the capacitance vessels is dependent to a greater extent on the vasoconstrictor fibres than on metabolites locally released during exercise

The main resistance vessels are precapillary and the main capacitance vessels postcapillary The deduced changes in capillary pressure indicate that the difference in response to sympathetic stimulation between resistance and capacitance vessels may hold for the whole pre and postcapillary sections The postcapillary resistance must be relatively more influenced than the precapillary during sympathetic stimulation *superimposed on exercise since the already high capillary pressure rose further during* the steady state phase of sympathetic stimulation This leads to an increased outpour of fluid into the tissues when activation of the vasoconstrictor fibres is added to exercise (Fig 1 3 and 5)

The general tendency of the resistance vessels to be dominated by the vasodilator metabolites and of the capacitance vessels to be dominated by the nervous supply has been described by Lewis and Mellander (1962) who studied the competition between metabolic influences during reduced blood upply to resting muscles exposed to constrictor fibre activation The present study shows that the result of this competition is the same when the normal relation between tissue metabolism and blood flow is disturbed not by reducing the blood flow but by increasing the metabolism

It is tempting to attribute a functional significance to the differentiated pattern of the response produced by the combination of exercise and increased vasoconstrictor fibre activity It seems appropriate that the amount of blood flowing through a muscle as well as the distribution of this blood within the muscle is dictated by the metabolic ds of the tissue while the amount of blood pooled in the muscle is directly ıfluenced by the sympathetic tone since the regional blood volume is the immediate ource of an increased venous return and an adequate central blood volume But it is obvious that such an arrangement could not be adequate if the differences in regulation of the resistance and the capacitance vessels were absolute since sudden situations may mpose such a stress on the general circulation as to necessitate reduction of the blood low even through exercising muscles The sympathetic system can apparently cope with such situations for shorter periods during which the exercising muscle can compensate such a reduction only by increasing the capillary surface area and thereby increasing the surface area available for exchange and decreasing the diffusion distances

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The Potassium Ion as a Vasodilator during Muscular Exercise

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Abstract

KJELLMER, I. *The potassium ion as a vasodilator during muscular exercise.* Acta physiol. scand. 1965. 63. 460—468. — Calf muscles of cats were perfused with blood at constant flow rates. Tissue volume, blood flow, arterial and venous pressures were measured. Exercise was imitated by intermittent contractions of the muscles. During contractions the potassium concentration of the venous plasma from the calf increased by up to 100 per cent. Potassium salts infused intra-arterially at low rates dilated the vessels. It was calculated that the potassium released during exercise directly explains 25 to 65 per cent of the dilatation during exercise, the percentage being smallest when dilatation was slightest. There is presumably a diffusion gradient for potassium between tissue and blood with consequent underestimation of the role of the potassium ions, particularly when the dilatations are only weak. Therefore 65 per cent probably comes closest to the true value of the proportion of the dilatation due to the potassium ions.

Potassium infusions produced the same vascular response as exercise: a decrease of flow resistance was accompanied by a proportionate increase of the capillary filtration coefficient without signs of any increase in capillary permeability or dilatation of the capacitance vessels. Potassium is the only dilative substance hitherto found to produce exactly the same response as exercise.

It has long been known that injections of small amounts of potassium ions dilate peripheral vessels. In his review of the vascular effects of potassium DAWES (1941) suggested that the hyperemia of exercise might be due to the action of potassium ions, for it was then known that potassium ions pass from the intra- to the extracellular space during muscle activity. It was this possibility, which does not seem to have attracted much attention, that prompted the present studies. Preliminary experiments (KJELLMER 1960, 1961) showed that tetanic contractions of skeletal muscle were accompanied by an efflux of potassium ions from the muscle to the venous plasma and that intra-arterial infusions of potassium salts produced vasodilatation. Comparison of the venous plasma potassium concentration after tetanus and during potassium infusions indicated that about one third of the vasodilatation during exercise can be explained by the increase in the potassium concentration.

These observations have now been followed up along two different lines. Firstly, to allow of quantitative comparison between the vasodilatation during exercise and the dilatation during potassium infusions, the dilatations were stabilized by rhythmic muscle contractions and by intra-arterial infusions during constant blood flow perfusion. Secondly, the effects of the two dilating procedures on different sections of the muscle vascular bed, i.e. resistance vessels, precapillary sphincters and capacitance vessels were compared. If potassium ions are responsible for a major part of the dilatation during exercise, infusions of potassium should reproduce the vascular pattern of exercise.

Methods

16 cats, weighing 2.6 to 4.5 kg, were anesthetized with ether and given either chloralose 70 mg/kg or nembutal 35 mg/kg i.v. The animals were tracheotomized and heparin 5 mg/kg was given i.v. The volume of blood sampled for the electrolyte determinations was substituted either by a low weight dextran Tyrode solution (Rheomacrodex®, Pharmacia) or by blood from a donor cat.

The preparation of the calf has been described recently (Kjellmer 1964). It consists almost only of calf muscles in circulatory connection with the rest of the body solely through the popliteal vessels. Blood flow was recorded with a photo-electric drop counter connected to the cannulated popliteal vein. Arterial inflow was regulated with a perfusion pump which directed the flow from the femoral to the popliteal artery. Proximal to the pump, solutions were infused into the perfusion tubing to ensure complete mixing. The systemic arterial pressure was measured with a Hg manometer connected with the contralateral femoral artery, and the pressure at which the perfusion was delivered by the pump was measured with another Hg manometer connected with the tubing distal to the pump. The volume changes of the calf were registered with a plethysmograph filled with water and a piston recorder.

The capillary filtration coefficient was obtained by raising the venous outflow pressure by 10 mm Hg for about 2 min and calculating the amount of fluid filtered per 100 g of tissue per min per mm Hg change of capillary pressure, assuming that 100 per cent of the applied venous pressure reached the capillaries during constant perfusion.

The sciatic nerve was divided and the peripheral end stimulated by bipolar silver electrodes connected with a Grass stimulator, Model S 4, set at 1–2 V, 0.1 msec and 0.5–2 imp/sec. Isotonic solutions of KCl (1.12 g/100 ml), KNO_3 (1.53 g/100 ml) and, as a control, NaCl (0.9 g/100 ml) were infused i.a.

In 5 experiments the effects, if any, from the adrenal medulla were eliminated by extirpating the right and denervating the left suprarenal gland.

For electrolyte analysis blood samples were taken in small, stoppered test tubes from the venous outflow tubing close to the muscle and occasionally also from the arterial perfusion tubing. Using an Eppendorf flame photometer double determinations were made of the potassium and sodium ion concentration in each plasma sample and the mean value was used. The difference between the duplicate determinations of potassium was 3.4 per cent of the mean value found for 45 pairs of samples (S.E. 0.18), the corresponding figures for sodium being 3.1 per cent and 102 pairs of samples (S.E. 0.37). For a normal cat plasma of 4.0 meq K/l and 150 meq Na/l the mean errors [illegible] were thus 0.14 meq/l and 4.7 meq/l respectively.

Samples were taken [illegible] period and during stable vasodilatation produced by exercise or potassium salt infusion. In 7 cats the dilatation was produced by both exercise and potassium infusion alternately, in [illegible] by exercise alone and in [illegible] by potassium only.

In 5 cats the potassium concentration in the red cells was calculated from blood samples, part of which was hemolyzed with an equal volume of distilled water, while another part was used for determination of the plasma potassium and the hematocrit. The concentration of potassium in the red cells was [illegible] less than [illegible] that in the plasma, which is in complete accordance with the [illegible] values published by Bernst[illegible] 1954, who found the quotient between the [illegible] concentration of potassium in cat blood to be 1.7. Hemolysis [illegible] these experiments [illegible] analyses on human blood

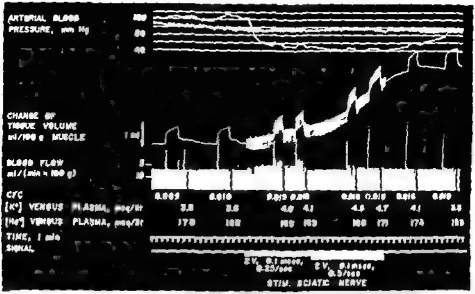


Fig 1 Cat 3 2 kg Effect of muscle contractions on pump pressure systemic arterial pressure tissue volume blood flow capillary filtration coefficient (CFC) and concentrations of potassium and sodium in venous plasma The systemic pressure persisted at about 100 mm Hg during the whole period while the pump pressure dropped during exercise The venous samples were taken immediately after each CFC-determination — the sampling periods are indicated by the black bars in the flow record

Results

Exercise Rhythmic contractions of the calf muscles produced a drop in vascular resistance This drop which was graded by adjustment of the frequency of muscle contractions was accompanied by an efflux of potassium from the muscles as evidenced by an unchanged arterial and a simultaneously elevated venous plasma potassium concentration The elevation of the potassium concentration in venous plasma was related to the severity of exercise and to the degree of vasodilatation Fig 1 shows a typical example where two different intensities of exercise were used Note that the potassium concentration increased more during the more intense exercise and that after exercise the concentration of potassium in the venous plasma returned towards control level largely at the same rate as the flow resistance Fig 1 also shows that exercise did not produce any consistent changes in the sodium concentration of venous plasma which was a typical finding

The change of the potassium concentration in the venous plasma in all the 11 expts with exercise are given in Fig 2 as mean values To enable comparison of the results obtained in several experiments both the vascular resistance and the increase of the potassium concentration are expressed in per cent of control values because the absolute values varied within one and the same experiment as well as between experiments Fig 2 shows the correlation between the rise of the potassium concentration in venous plasma and the degree of vasodilatation During the more severe exercise when the flow resistance was minimal the potassium in the venous plasma rose to double the control value

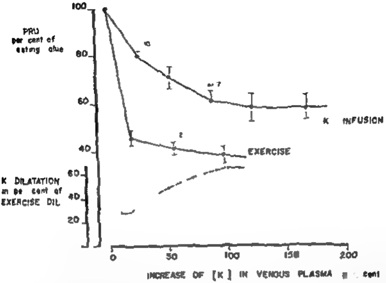
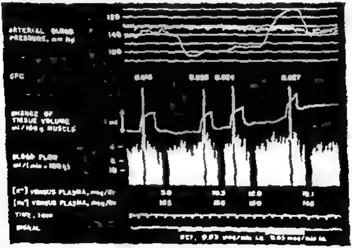


Fig 2 Relation between peripheral resistance and change of potassiu n concentrat on in venous plasma during infusion of potass um salts (12 expts) and exercise (11 expts) The values were classed according to changes in potassium concentration Mean values are shown Th verticals denote ± 1 S E n = number of determinations in each class The lower part of the figure gives the potassium dilatation in per cent of exercise dilatation i e the fraction of the exerc se dilatation explained by the released potassium ions.



F g 3 Cat 2 9 kg Effects of intra arterial infusions of isotonic KCl at two different rates on system c rterial p essure pump p essur cap llary filtration coefficient (CFC) tissue volume, blood flow and the con entrat ons of potassium and sodium n enous plasma The systemic pressure rema ned about 160 mm Hg during the whole period while the pump pressure dropped at the lower and rose at the h gher rate of nfus on

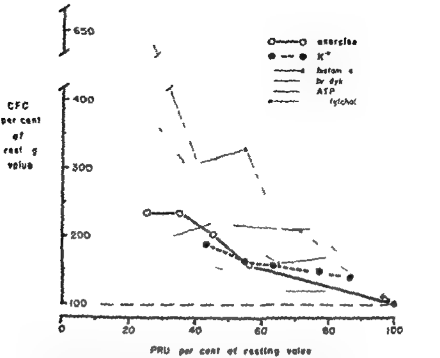


Fig. 4. Relation between increased CFC and decreased peripheral resistance induced by exercise (32 determinations) and by infusion of potassium salts (30 determinations). The results obtained with the other substances were taken from Kjellmer and Odelram 1965.

Potassium infusions. When isotonic KCl was infused intra-arterially, flow resistance decreased in proportion to the increase of the venous potassium concentration. The most pronounced dilatations were induced by infusions that raised the potassium concentration in the venous plasma to about 15 or 20 meq/l. These dilatations were only occasionally maximal — usually the vessels could be dilated further by other dilating agents or by exercise. Infusion of still more KCl produced not further dilatation but vasoconstriction. The reversal from dilatation to constriction usually occurred at a potassium concentration of 20—30 meq/l. Fig. 3 shows an example in which KCl infused at a low rate decreased, and at a higher rate increased, vascular resistance. The same results were obtained when KNO_3 was used instead of KCl. Control infusions with 0.9 per cent NaCl produced no such effects except at the highest rates of infusion, when a slight vasodilatation was recorded. At such high rates potassium infusions caused vasoconstriction. During infusions of potassium at the highest rates the sodium concentration of the venous plasma fell by 10—15 per cent.

Exclusion of the adrenals did not affect the potassium efflux from the contracting muscles or the vascular response to infusions of potassium salts. Sympatholytic drugs (dihydroergotamine, phenoxybenzamine) in doses sufficient to block the constrictor response to stimulation of the sympathetic nerves did not affect the vasodilatation following small doses of potassium or the constriction following larger doses.

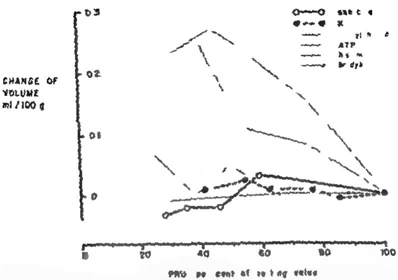


Fig 5 The relationship between change of tissue volume due to variation of regional blood volume (capacitance changes) and decreased peripheral resistance induced by exercise (15 determinations) and by infusions of potassium salts (31 determinations) The results obtained with the other substances are taken from Kjellmer and Odelram 1965

The pooled results from all 12 expts where potassium salts were infused are also shown in Fig 3 The relationship between the degree of vasodilatation and the change of potassium concentration in the blood gives the expected dose response curve with the convexity towards the response axis

The extent to which the potassium ions released during exercise were responsible for the exercise vasodilatation was calculated from the relation between the change of vascular resistance and increase of potassium concentration in venous blood during potassium infusions respectively during graded exercise This proportion was found to vary from about 25 per cent at the smallest dilatations to 65 per cent during the periods of most intense exercise (Fig 2)

Effects on various consecutive vascular sections in the skeletal muscle The effects of exercise have been reported in detail previously (Kjellmer 1964 Kjellmer and Odelram 1965) but will be described again briefly to enable comparison with the dilatation caused by potassium Fig 1 shows that exercise decreased the flow resistance and increased the capillary filtration coefficient (CFC) but had no appreciable effect on the capacitance vessels as evidenced by the absence of any increase in tissue volume during the phase of decreasing vascular resistance The findings illustrated in Fig 1 hold true for the whole series of experiments They are summarized in Fig 4 which shows the relation between change in vascular resistance and change in CFC during exercise and in Fig 5 which gives the relation between the change of vascular resistance and change of tissue volume due to variation of vascular capacitance during exercise

The infusion of potassium salts also increased the CFC in proportion to the degree of vasodilatation Fig 3 gives an example of CFC during control conditions and during dilatation Fig 4 shows the results for all potassium infusions It is clear that the relation

between decrease of flow resistance and increase of CFC during potassium infusions was largely the same as that during exercise

During potassium infusions only small inconsistent tissue volume changes that could be ascribed to dilatation or constriction of capacitance vessels were noted (*cf* Fig 3) Fig 5 gives the relation between change of tissue volume due to capacitance changes and decrease of flow resistance during potassium infusions and here too it can be seen that the relations are the same as those found for exercise

When the potassium concentration was increased to reversal point *i e* when the vessels reacted with constriction instead of dilatation the CFC remained high or increased further in those 5 expts where it was determined during the constrictor phase An example of this is given in Fig 3 The vasoconstriction did not influence the capacitance vessels enough to produce any discernible change in the tissue volume

Discussion

In 1880 Gaskell suggested that the hyperemia of exercise is due to the local release of some factor from the muscle Despite extensive research it has however, not been possible to find any one substance capable of explaining such dilatation of the vessels (for reviews see Hilton 1962 and Barcroft 1963) As a working hypothesis it therefore appeared reasonable to ascribe the phenomenon to the joint effect of two or more substances The next step was then to assess what proportion of the dilatation can be explained by substances proposed by earlier workers In the present investigation interest was focused on only one of these substances namely potassium When the role played by potassium was calculated by comparing the dilatation produced by exercise with that caused by a potassium infusion that increased the potassium concentration to the extent as did the exercise the fraction explained by the released potassium ions ... ried between 25 and 65 per cent (Fig 2)

The vasodilatation following the infusion of potassium must have been due to the potassium ion since KCl and KNO produced the same effect The decrease in sodium concentration following the infusions cannot explain the dilatation — such a decrease tending if anything to constrict the vessels (*cf* Friedman and Friedman 1963) However the changes in sodium concentration were probably much too small to affect vascular tone at all (*cf* Overbeck Molnar and Haddy 1961)

It can thus be concluded that potassium ions are responsible for a substantial proportion of exercise hyperemia especially since this type of comparison may underestimate the importance of the potassium ions for the following reasons If it is assumed that the arteriolar smooth muscle cells react not to the concentration of compounds in the actual plasma but rather to the concentration of compounds in the fluid in which they are bathed *i e* in the interstitial fluid the concentration of potassium in the venous plasma is lower than the relevant concentration value during exercise when potassium diffuses from the cells to the interstices and to the plasma but is higher when potassium moves in the opposite direction following infusion of potassium salts

The extent of this underestimation depends directly on the actual concentration gradient between tissue and blood but this gradient cannot be determined at present However in studies on the kinetics of potassium exchange between blood and skeletal muscle Renkin (1959) who used tracer amounts of K^{42} added to the arterial supply of artificially perfused gracilis or gastrocnemius muscles of dogs showed that at an extremely low rate of blood flow the extraction of K^{42} approached 100 per cent but

decreased at higher flow rates to become 47 per cent at 10 ml/(min × 100 g) (calculated from Renkin's table 2). Since the blood flow in the present experiments was 5—10 ml/(min × 100 g) the diffusion of potassium was presumably not fast enough to produce an equilibrium between tissue and blood.

It was thought that lymph might reflect the tissue potassium better than venous blood. But lymph samples from the calf muscles were found to contain the same concentration of potassium as venous blood. However, at the low lymph flows in this preparation potassium was exchanged so rapidly across the lymph vessel wall that any concentration gradient was equilibrated during the passage along the lymph vessel (Jacobsson and Kjellmer 1964). Cardiac lymph, on the other hand, which flows at a much higher rate has recently been shown in a heart lung preparation to have a potassium concentration some 50 per cent higher than that of coronary sinus blood (Areskog, personal communication), which indicates that at least in this particular muscle there is no diffusion equilibrium between tissue and venous blood.

A substantial concentration gradient would result in underestimation of the role played by potassium in exercise hyperemia. For two reasons the error should be greatest at the lowest potassium concentrations. Firstly, a given gradient will affect the calculated result most when the potassium concentration corresponds to the steepest part of the dose response curve for potassium and vasodilatation and, secondly, the diffusion distance will be longest during rest and at the lowest degrees of vasodilatation, when only a small fraction of the capillaries are open to blood flow. According to this line of thought, the fraction of exercise hyperemia explained by released potassium ions should be smallest at the lowest potassium concentrations, which also proved to be the case (Fig. 2). Therefore the true fraction probably lies closer to 65 per cent than to 25 per cent.

Comparison of the patterns of vasodilatation produced by the two procedures gives further support to the assumption that potassium is responsible for a substantial proportion of the vasodilatation during exercise. Both exercise and potassium ions dilate the resistance vessels with an increase of the CFC while the capacitance vessels remain largely unaffected. In similar experiments Kjellmer and Odelram (1965) found acetylcholine, ATP, histamine and bradykinin to produce a response differing from that of exercise: acetylcholine and ATP dilated the capacitance vessels, while histamine and bradykinin produced signs of increased capillary permeability, as reflected by very high CFC values together with the production of irreversible tissue edema. Neither exercise nor potassium dilatation was accompanied by these signs of increased permeability. The relationships between the changes in vascular resistance and in CFC respectively in vascular capacitance during exercise and potassium infusions in the present experiments are shown in Fig. 4 and 5, which for comparison also include the effects of the other vasodilators used in the abovementioned study. So far, the potassium ion is the only dilator that has produced a response closely resembling that of exercise.

The effect of potassium always varied with the dose infused, small doses producing dilatation, large ones constriction (Fig. 3). One might therefore wonder whether the amount of potassium released during severe exercise might not be able to convert the dilatation into constriction. Emanuel, Scott and Haddy ([illegible]) have, however, presented evidence that potassium induced constriction is confined to the larger arteries (above 0.5 mm), while the arterioles remain dilated. Bohr and Goulet (1961) in in vitro studies also found large vessels and arterioles to differ in the response to an increased potassium concentration. The finding in the present series that CFC remained increased

— *i e* that precapillary sphincters remained open — despite strong vasoconstriction may therefore be regarded as evidence that the small vessels are dilated and the large arteries constricted by an increase of potassium concentration — the constriction having a higher threshold This might mean that the vessels that react to potassium with constriction are situated outside the muscle tissue and are thus not directly influenced by changes in concentrations of tissue potassium Moreover constriction occurs only at such high potassium concentrations as probably never normally occur during exercise

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Acta physiol scand 1965 63 469—471

From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

A Strychnine-Resistant Postsynaptic Inhibition in the Spinal Cord

By

JAN OLOF KELLERTH

Received 3 August 1964

Abstract

Kellerth J O *A strychnine resistant postsynaptic inhibition in the spinal cord* Acta physiol scand 1965 63 469—471 — In cats anaesthetized with pentobarbitone lumbar motoneurones were impaled with potassium citrate microcapillaries The inhibitory influence from muscle stretch receptors on the motoneurones was studied Attention was paid to the following criteria 1) monosynaptic excitability 2) firing rate during stimulation by depolarizing transmembrane current, 3) synaptic activation noise and 4) shift of average membrane potential A postsynaptic inhibition is described which is insensitive to convulsive doses of strychnine This strychnine resistant postsynaptic inhibition was frequently found So-called presynaptic inhibition was never observed

Since the first investigation of strychnine reversal (Owen and Sherrington 1911) many attempts have been made to elucidate the mechanism by which strychnine affects the reflex activity in the spinal cord

According to current views (Eccles 1964) strychnine very effectively eliminates the postsynaptic inhibitory actions in the spinal cord leaving only the so-called presynaptic inhibition intact Picrotoxin on the other hand diminishes the effect of presynaptic inhibition but has no direct influence on the postsynaptic membrane (Eccles Schmidt and Willis 1963) This difference in pharmacological action is supposed to be related to the occurrence of two separate types of inhibitory transmitters one acting presynaptically and the other postsynaptically

Strychnine resistant postsynaptic inhibition has been described in e g hippocampus and cerebellum (Andersen *et al* 1963) but its existence in the spinal cord has been considered to be doubtful The experiment to be reported demonstrates postsynaptic inhibition from stretch receptors on motoneurones which is resistant to strychnine

In adult cats anaesthetized with pentobarbitone and paralysed with gallamine triethiodide (Flaxedil) lumbar motoneurones were impaled with 3 M potassium citrate microcapillaries A bridge circuit similar to the one described by Araki and Otani (1955) was arranged for the simultaneous recording of trans membrane voltage changes

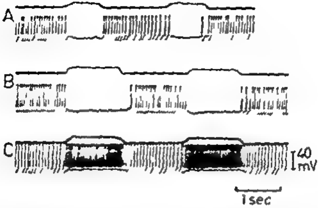

Fig 1 Strychninized cat Popliteal motoneurone (monosynaptic spike size 65 mV) during stimulation with depolarizing trans-membrane current Figure shows two 500 g pulls of the semitendinosus muscle (A) the tibialis anterior (B) and the triceps surae (C)

and passing polarizing currents through the microcapillary Stretch could be applied to the carefully freed semitendinosus triceps surae and tibialis anterior muscles by loading them with weights across a pulley connected to a myograph

The cats were given intravenous injections of 0 2 mg strychnine/kg

Only such motoneurones were used as responded by continued repetitive firing to a long lasting depolarizing current

A number of motoneurones were found whose behaviour during stretch of the different muscles was analyzed by the aid of the following criteria

1) monosynaptic excitability
2) firing rate during stimulation by trans membrane depolarizing current
3) synaptic activation noise and
4) shift of average membrane potential

The work by Granit Kellerth and Williams (1964 a b) shows that with natural stimuli such as muscle stretch only criterion 2 (stimulation by trans membrane current) is generally reliable in revealing changes of excitability while the other criteria can be misleading The synaptic activation noise is sometimes absent but when present it is an important sign of engagement of the postsynaptic membrane

Fig 1 shows records from a popliteal motoneurone (monosynaptic spike size 65 mV) after the administration of 0 2 mg strychnine/kg The neurone is set to fire repetitively by a continuous depolarizing current In A the upward deflection of the myograph indicates stretch of the semitendinosus muscle which inhibits the discharge When the load is released the firing recommences In B a strong inhibition is induced by stretch of the tibialis anterior while pulling on the triceps surae in C increases the rate of firing In both A and B the muscle stretches caused a hyperpolarization of the order of 2—3 mV which was only seen in connection with the depolarizing current and hence not fully reliable The size of the monosynaptic EPSP of this motoneurone showed a decrease by 10 during pull of either semitendinosus or tibialis anterior Stretch of tibialis anterior or triceps surae was accompanied by a marked increase of synaptic noise indicating an activation of the postsynaptic membrane

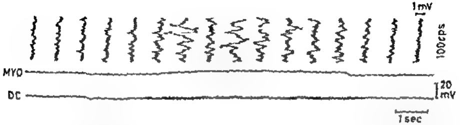

Fig 2 Strychninized cat Same motoneurone as in Fig 1 The picture shows one 500 g pull of the tibialis anterior On the fast AC-sweeps the "synaptic activation noise" and on the continuous DC-recording the average level of the membrane potential

Fig 2 from the same motoneurone shows on the AC-sweeps the increase in "noise" activity during the pull on tibialis anterior that proved to be inhibitory by the tests of Fig 1 The continuous DC recording in this case reveals no change of average membrane potential level No "activation noise" was observed during stretch of the semitendinosus muscle possibly meaning that the engaged synapses were too remote to be detected by the micro-electrode

In the popliteal peroneal and hamstring motoneurones investigated in this work strychnine resistant postsynaptic inhibition has proved to be a common finding In no case was it necessary to explain the results on the basis of presynaptic inhibition. Use of stimulation by transmembrane current always located the inhibition to the activated cell membrane

This work will be continued

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Acta physiol scand 1965 63 472—478

From the Department of Physiology University of Lund Sweden

In Vitro Glucose Metabolism in Different Tissues of Hypophysectomized Rabbits

By

DORA JACOBSOHN, S LARSSON and A NORGREN

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Abstract

Jacobsohn D S Larsson and A Norgren *In vitro glucose metabolism in different tissues of hypophysectomized rabbits* Acta physiol scand 1965 *63* 472—478 — The effect of hypophysectomy on the fate of uniformly labelled ^{14}C glucose was studied in cerebral cortex anterior and posterior hypothalamus adrenal cortex and thyroid gland of rabbits Determinations were also made of DNA content and oxygen consumption of the tissues The findings after hypophysectomy were as follows Oxygen uptake of all brain parts increased The $^{14}CO_2$ production of the posterior hypothalamus was increased Oxygen consumption and $^{14}CO_2$ production decreased in adrenal cortex and thyroid gland The formation of free ^{14}C alanine in the cerebral cortex decreased The formation of ^{14}C aspartic acid in the posterior hypothalamus increased In the adrenal cortex and thyroid gland a decreased conversion of ^{14}C glucose into all metabolites studied was found. The rate of formation of radioactive protein bound amino acids in the brain parts remained unchanged In the proteins of adrenal cortex and thyroid gland hardly any radioactivity could be detected The changes appeared to be closely correlated with the activities in various enzyme systems observed previously

Hypophysectomy in rats and rabbits was observed to be followed by a decreased activity of the enzymes involved in the metabolism of glucose (Jacobsohn Larsson and Norgren 1964) The decrease in enzyme activity was found in liver and skeletal muscle as well as in the thyroid gland and adrenal cortex Whether the changes observed were due to a decrease of a single rate limiting enzyme remains to be studied In rats with alloxan diabetes Fitch and Chaikoff (1962 found changes in the activities of a considerable number of enzymes in the liver The authors mentioned did not obtain support for the assumption that general changes in enzyme systems are due to a single bottle neck enzyme

A change in activity of enzymes involved in the metabolism of glucose would seem to be reflected in the fate of glucose incubated *in vitro* with tissue slices The fate of ^{14}C labelled glucose was found to vary in different tissues (Beloff Chain *et al* 1955 Hellman Larsson and Westman 1961 *a* and *b* Herdenstam 1962) Different parts of the brain also showed variations in the *in vitro* metabolism of glucose (Chain Larsson and Pocchiari 1960a and Andersson Larsson and Pocchiari 1961

The present study was performed in order to see whether correlations could be found between the effect of hypophysectomy on enzyme activities (Jacobsohn *et al.* 1964) and the *in vitro* metabolism of glucose.

Material and methods

25 young adult rabbits of both sexes were used. The females were virgins. All rabbits were obtained from the same breeder. The animals had free access to a pellet diet (Fors Ltd) and tap water. After their arrival at the Institute the animals received sulfa pyrimidine (Rigesol, Ferrosan Ltd) with the tap water in order to counteract coccidiosis.

Hypophysectomy was performed in 12 animals by the parapharyngeal route (Jacobsohn and Westman 1940). Details concerning the control of completeness of hypophysectomy as well as the evaluation of the material may be found in a previous publication (Jacobsohn *et al.* 1964).

3 weeks or more after hypophysectomy the animals were killed by decapitation and as quickly as possible tissues were removed for study: a) *cerebral cortex* adjacent to the sagittal sinus and anterior to the central gyrus; b) *anterior hypothalamus*, that is an area limited anteriorly by the caudal margin of the optic chiasma and posteriorly by a mid section through the infundibulum, laterally by the medial margin of the columna fornix descendens. The thickness of the piece of tissue was the distance from the basal surface of the brain to the ventro-medial thalamic nuclei; c) *posterior hypothalamus* involving the tissues posterior to the former part and reaching the rostral parts of the mesencephalon; d) *adrenal cortex* dissected free from medullary tissues of both adrenals; e) *thyroid gland* cleaned of fat and connective tissues. Microscopic examinations of sections through parts of the sample were performed when it appeared desirable clearly to identify the structure of the tissues. All samples were cut into about 0.2 mm thick slices (Chain *et al.* 1960a). The slices were transferred into Warburg vessels containing 0.5 ml of ice chilled incubation medium. The incubation medium contained 0.1 [illegible] glucose with a total radioactivity of 10 µC per Warburg vessel. Uniformly [illegible]C labelled glucose was obtained from the Radiochemical Centre, Amersham, England. The radioactive glucose was diluted with non radioactive substrate to give a specific activity of 25 µC/mg. The incubation medium contained a sodium potassium phosphate buffer with the composition described by Chain *et al.* (1960b). The centre wells of the vessels contained rolls of filter paper soaked with 30 [illegible] KOH for the absorption of respiratory CO_2. The incubation was performed in oxygen for 60 min at 37 °C. Oxygen consumption was measured every 15 min. After incubation the formed K_2CO_3 was collected with $BaCl_2$ as described by Villee and Hastings (1949) and the radioactivity measured as $BaCO_3$.

After incubation the tissues were homogenized and extracted. The radioactive metabolites were separated by paper chromatography as described by Chain *et al.* (1960b) and Hellman and Larsson (1961 *a*). The insoluble residues obtained were pooled two and two and then hydrolyzed as described by Larsson, Hellman and Carstensen (19[illegible]). All chromatograms were scanned quantitatively by a modification of the device designed by Frank *et al.* (19[illegible]).

Parts of the tissues were preserved for DNA determination. DNA was extracted as described by Schneider (1945) and determined according to Dische (1930) as modified by R[illegible] (1952).

Results

DNA. Table I shows the DNA values for the various tissues in intact and hypophysectomized rabbits. The DNA content in adrenal cortex and thyroid gland was significantly increased after hypophysectomy.

Oxygen uptake and CO_2 production. The oxygen uptake of the brain parts studied increased significantly after hypophysectomy (Table II) regardless of whether the values were expressed per unit wet weight or per unit DNA. In adrenal cortex and thyroid gland hypophysectomy resulted in a decreased oxygen uptake. After hypophysectomy the CO_2 production was significantly increased in the posterior hypothalamus but not in the cerebral cortex and anterior hypothalamus (Table III). The (A) production in adrenal cortex and thyroid gland decreased significantly after hypophysectomy.

TABLE I DNA content (mg per g wet weight) in different tissues of intact and hypophysectomized rabbits Mean ± S E M (number of observations) Differences between intact and hypophysectomized rabbits have been calculated according to Student's t test. a P < 0 001

	Cerebral cortex	Ant hypothal	Post. hypothal	Adrenal cortex	Thyroid gland
Intact	[illegible] ± 0 11 (12)	[illegible] ± 0 33 (13)	6 9 ± 0 50 (10)	5 2 ± 0 44 (13)	7 [illegible] ± 0 50 (11)
Hypophysectomized	2 6 ± 0 13 (12)	[illegible] 8 ± 0 49 (12)	7 2 ± 0 6[illegible] (10)	12 3 ± 1 68 (12)a	11 0 ± 0 6[illegible] (11)a

TABLE II Oxygen consumption and $^{14}CO_2$ production in different tissues of intact and hypophysectomized rabbits Results expressed as µl of oxygen uptake and glucose converted into CO in [illegible] mg of wet tissue per unit DNA after 1 hr incubation at 37 C in O in 0 [illegible] ml medium Glucose concentration 0 1 Total radioactivity per vessel = 10 µC. Mean ± S E.M The differences between intact and hypophysectomized rabbits have been calculated according to Student's t test P 0 001 b P < 0 01 P < 0 0[illegible]

	O_2	CO	Number of samples
Cerebral cortex			
Intact	1[illegible] 2 ± 0 [illegible]0	11 1 ± 0 [illegible]3	1[illegible]
Hypophysect	1[illegible] 2 ± 0 [illegible]	11 6 ± 0 8[illegible]	[illegible]
Anterior hypothalamus			
Intact	[illegible] 8 ± 0 38	5 6 ± 0 33	13
Hypophysect	[illegible] 6 ± 0 0[illegible] b	4 6 0 38	[illegible]
Posterior hypothalamus			
Intact	4 9 ± 0 38	3 4 ± 0 24	10
Hypophysect	[illegible] 2 ± 0 43	4 7 ± 0 31	10
Adrenal cortex			
Intact	4 3 0 4[illegible]	[illegible] 0 0 18	13
Hypophysect	1 4 0 19	0 7 0 10	[illegible]
Thyroid gland			
Intact	1 0 17	0 [illegible] 0 09	11
Hypophysect	1 1 0 1[illegible] b	0 3 0 0[illegible]	11

Lactic acid As may be seen from Table III hypophysectomy did not change the rat of C lactic acid formation from the substrate glucose in any of the brain parts studied On the other hand hypophysectomy caused a significant decrease in the rate of C lactic acid formation in adrenal cortex and thyroid gland

TABLE III Effect of hypophysectomy on the conversion of ^{14}C glucose into lactate and amino acid in different tissues of rabbits Results expressed as μ glucose converted in 25 mg of wet tissue per unit DNA after 1 hr incubation at 37 C in O in 0.5 ml medium Glucose concentration 0 1 Total radioactivity per vessel = 10 μC Mean $\pm$ S E M The differences between intact and hypophysectomized rabbits have been calculated according to Student's t test ^{a}P < 0 001 ^{b}P < 0 01 P < 0 05 Number of samples from different animals see Table II

	Lactic acid	Asp acid	Alanine	Glutam. acid	Glutamine	GABA
Cerebral cortex						
Intact	48 7±5 50	0 52±0 051	0 36±0 061	4 25±0 27	1 77±0 161	0 80±0 0 ?
Hypo-physect.	48 1±5 92	0 43±0 050	0 33±0 062$_b$	4 24±0 36	1 56±0 176	0 68±0 034
Anterior hypothalamus						
Intact	30 6±2 71	0 21±0 070	0 18±0 013	1 0±0 15	0 34±0 041	0 99±0 017
Hypo-physect.	27 7±2 81	0 20±0 077	0 16±0 013	1 51±0 16	34±0 043	0 98±0 013
Posterior hypothalamus						
Intact	24 7±1 98	0 15±0 007	0 14±0 015	1 00±0 040	0 13±0 010	0 73±0 081
Hypo-physect.	26 4±2 65	0 19±0 013	0 13±0 013	1 16±0 110	0 25±0 076	0 88±0 100
Adrenal cortex						
Intact	4 6±0 24	0 12±0 007	0 04±0 003	0 16±0 012	0 07±0 008	
Hypo-physect	2 6±0 22	0 05±0 005	0 07±0 002	0 08±0 009	0 04±0 005	
Thyroid gland						
Intact	? 1±0 18	0 03±0 003	0 14±0 015	0 17±0 018	0 04±0 005	
Hypo-physect	1 3±0 17$_b$	0 07±0 003	0 10±0 014	0 09±0 010$_b$	0 03±0 003	

α-amino acids In the cerebral cortex of hypophysectomized rabbits the formation of C alanine decreased (Table III) The anterior hypothalamus seemed to be unaffected by hypophysectomy with regard to the formation of any of the amino acids formed from the substrate glucose In the posterior hypothalamus hypophysectomy resulted in an increased C aspartic acid formation The rate of formation of labelled α amino acids in the adrenal cortex and thyroid gland decreased after hypophysectomy

Glutamine and γ-aminobutyric acid (GABA) After hypophysectomy C glutamine formation from the substrate glucose was increased in the posterior hypothalamus (Table III) No change in C GABA formation was found after hypophysectomy in any of the brain parts studied After hypophysectomy C glutamine formation decreased in the adrenal cortex but not in the thyroid gland

Hydrolyzed insoluble residues Table IV shows that hydrolyzed insoluble residues from the various tissues of intact animals contained C labelled amino acids After hypo-

TABLE IV. Effect of hypophysectomy on the conversion of ¹⁴C glucose into protein bound amino acids. Results expressed as in Table III. Number of pooled samples of different tissues = 4

Tissue	Asp acid	Alanine	Glut acid	Glutamine	Leucine iso leucine	Proline
Cerebral cortex						
Intact	0.03 ± 0.005	traces	0.41 ± 0.031	0.12 ± 0.016	0.14 ± 0.020	
Hypophysect.	0.02 ± 0.005	traces	0.43 ± 0.052	0.13 ± 0.017	0.14 ± 0.022	
Ant. hypothalamus						
Intact	0.11 ± 0.018	traces	0.23 ± 0.023	0.06 ± 0.007	0.15 ± 0.017	
Hypophysect.	0.12 ± 0.016	traces	0.20 ± 0.077	0.06 ± 0.008	0.16 ± 0.018	
Post. hypothalamus						
Intact	0.11 ± 0.017	traces	0.22 ± 0.027	0.02 ± 0.001	0.12 ± 0.018	
Hypophysect.	0.10 ± 0.012	traces	0.19 ± 0.026	0.03 ± 0.004	0.11 ± 0.019	
Adrenal cortex						
Intact	0.02 ± 0.003	0.03 ± 0.003	0.04 ± 0.003		0.02 ± 0.003	0.08 ± 0.009
Hypophysect.	traces	traces	traces		traces	traces
Thyroid gland						
Intact	0.02 ± 0.004	0.05 ± 0.006	0.05 ± 0.004	traces	0.10 ± 0.014	
Hypophysect.	traces	traces	traces		0.01 ± 0.001	

physectomy the incorporation was generally decreased in the adrenal cortex and thyroid gland, but seemed to be unchanged in the various parts studied of the brain.

Discussion

The present work on hypophysectomized rabbits revealed an increase of the oxygen uptake in three different parts of the brain. This might be assumed to be due to a hightened level of the general metabolism of brain tissues. An increased metabolism usually involves an increase in the utilization of exogenous substrates, in the present study this would concern the labelled glucose medium. Amongst other things, increased CO_2-values should reasonably be expected then. As may be seen from Table II the CO_2-values were not elevated after hypophysectomy. When "exogenous" RQ values (CO_2/O_2) were calculated it actually turned out that the utilization of exogenous glucose decreased after hypophysectomy in the cerebral cortex and the anterior hypothalamus. Andersson *et al.* (1961) found that the RQ of the various parts of the brain was close to 1. In the present study the true RQ was not measured. Although unlikely, a changed RQ after hypophysectomy cannot be excluded.

The observation of an increased oxygen uptake in the brain after hypophysectomy

agrees with previous findings of others Gordan (1956) who studied the arterio-venous differences of oxygen observed an increased oxygen uptake of the total brain tissue in hypophysectomized patients Reiss and Rees (1947) found in rats that the hexokinase activity of suspensions made from the grey matter of the brain as well as the anaerobic glycolysis of slices from the same parts increased after hypophysectomy

In the posterior hypothalamus the utilization of exogenous glucose appeared slightly increased after hypophysectomy The conversion of C glucose into C aspartic acid and glutamine was also increased Furthermore the observations agree with a previous study showing that the activities of lactic dehydrogenase and glutamic oxalacetic transaminase in a part of the hypothalamus corresponding to the posterior hypothalamus increased after hypophysectomy (Jacobsohn *et al* 1964) The findings seem to be of interest with regard to the tendency of hypophysectomized rabbits to succumb to hypoglycaemia

With regard to differences in the utilization of exogenous glucose in different parts of the brain it should be mentioned that Forssberg and Larsson (1954 and 1955) and Anand *et al* (1961) in metabolic studies observed that different areas of the hypothalamus did not respond equally to physiological stimuli such as hunger and satiety

In the brain of animals with intact pituitary gland the incorporation of C glucose into C amino acids was found to be rather high (Chain *et al* 1960a and Gaitonde Marchi and Richter 1964) The major portion of the amino acids formed in the brain notably in the cerebral cortex by the conversion of C glucose consists of glutamic acid In view of the presence of active transaminase and of the relatively large glutamate pool in the brain this does not seem surprising (Krebs Eggleston and Hems 1949) In the hypothalamus a large proportion of the amino acids formed from the substrate glucose was found as GABA the decarboxylation product of glutamic acid (Table III

For the adrenal cortex and the thyroid gland both of which atrophy considerably after hypophysectomy it appeared appropriate not to express values on a wet weight basis but per unit DNA (cf Jacobsohn *et al* 1964) As in the previous study the DNA content in the adrenal cortex and thyroid gland was in the present work found to be significantly increased after hypophysectomy (Table I After hypophysectomy the two endocrine organs presented a decreased overall metabolism of glucose including decreased formation of amino acids from the substrate glucose This agrees with previous observations of decreased activities of the enzymes participating in the metabolism of glucose as well as of α amino acid transaminases Jacobsohn *et al* 1964 According to Rosen and Nichol (1963 the importance of transamination reactions lies in the capability to catalyze the interconversion of amino acids and α keto acids which are carbohydrate precursors

Hypophysectomy did not change the amount of C incorporated into the proteins of the brain regions studied In the adrenal cortex and thyroid gland the rate of metabolism was so depressed that after hypophysectomy only traces of C could be found in the proteins This is in line with the findings of decreased conversion of C-glucose into free amino acids in these organs From the total record it would seem that the modifications observed previously after the removal of the rabbit's pituitary gland in enzyme systems are closely related to changes concerning the [illegible] metabolism of glucose

Our thanks are due to Chem ing S A Jonsson and Misses Lilla Hoff Clary Nykvist and Eva Wingren for skilful and devoted technical assistance The expenses of this work were in part defrayed by grants (L 18 and L 493 from the Swedish Medical Research Council

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Acta physiol. scand. 1965. 63. 479—486

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Responsiveness of the Erythron to Variations of Oxygen Tension in the Chick Embryo and Young Chicken

By

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Abstract

JALAVISTO, E., I. KUORINKA and M. KYLLÄSTINEN. *Responsiveness of the erythron to variations of oxygen tension in the chick embryo and young chicken.* Acta physiol. scand. 1965. *63*. 479—486. — The reactivity of the chick embryo erythron to varying ambient oxygen concentrations was investigated. The hemoglobin (Hb) concentration, the hematocrit and red cell count were determined in embryos incubated in 40 % O_2, air and 15 % O_2. The age of the embryos varied between 9 and 19 days. The results indicated that embryos weighing less than 7 g and approximately less than 14 days old do not increase their Hb, hematocrit or erythrocyte count when exposed to 15 % O_2. In embryos aged 16 days or over the 15 % O_2 exerts a stimulatory effect: the Hb and hematocrit values exceed those found in the embryos incubated in 40 % O_2. The mean corpuscular volume and the mean corpuscular Hb are not affected by differences in ambient oxygen concentrations.

It is pointed out that the 15th day of incubation seems to represent a critical phase in chick embryo development — it is not only approximately the date when myeloid erythropoiesis begins to dominate, but also the date when, for instance, some polyamines begin to be synthetized and the hypophysis becomes functionally active.

The mechanism responsible for the regulation of the red cell production is still to a certain extent obscure. It is well known that anoxic exposition, bleeding and administration of cobalt give rise to humoral agents, the so-called erythropoietins, which stimulate the production of red cells. The main site of production of the erythropoietin seems to be the kidney, but the renal erythropoietin is under these conditions not the sole erythropoiesis stimulating factor (Rosse and Waldman 1962, Piha 1962). However, the mechanism by which the erythropoietins are formed and their mode of action is not clear (for references, Jacobson and Doyle 1962, Stohlman 1962, Remmele 1963). The nature and role of the accessory factors are even less known.

In a previous paper (Astola *et al.* 1959) it was shown that in the early chick embryo incubated at different oxygen tensions the total hemoglobin (Hb) was in a constant relation to embryo weight irrespective of the oxygen tension. The sensitivity of the Hb

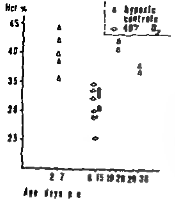

Fig. 1. Hematocrit values in chicken reared at various oxygen tensions. On the abscissa the first and last day of exposure in the different experimental groups.

forming system to variations in oxygen tension thus seems to develop during the ontogeny. The purpose of the present paper was to investigate more closely the phase during the embryonic development which corresponds to the appearance of responsiveness to a reduction in the oxygen supply. It was thought that if a limit can be established it might be possible to relate it to some definite developmental stage in the embryo. It would then perhaps be possible to obtain some hints as to the system involved in the adult organism as well.

Material and methods

White Leghorn eggs were incubated in air tight wooden boxes and ventilated through two tubes (inlet and outlet tubes situated diagonally at opposite corners of the boxes). The ventilation rate was adjusted with rotameters to 200 ml/min. On the bottom of the boxes was a free water surface, but the ventilation maintained the moisture at about 80—89 per cent. The incubation temperature was 39.0. Three ventilating gas mixtures were used: 15 % O_2 in N_2, air and 40 % O_2 in N_2, the last mentioned acting as control since according to Grux and Romanoff (1944) 40 % O_2 represents the optimum concentration for growth of the embryo.

The control and the experimental eggs were always incubated simultaneously and belonged to the same batch of eggs. This is absolutely necessary, because there seem to exist seasonal differences in, for instance, the level of Hb, erythrocyte count, etc. In spring the hematological values have a slight tendency to increase.

The hematological determinations were begun after 9 days incubation. On each of the following days up to 18 days (occasionally to 19 days) some (6–7) eggs were removed for analysis. The arteries and veins were located and blood samples drawn from the great vessels. The hematocrit was determined with 4 min centrifugation in heparinized capillaries at 11 500 rpm which corresponds to an average acceleration of 6 000 g (IEC Micro-Capillary centrifuge). Hb was determined by photometry in an alkaline (Na_2CO_3) solution as oxyhemoglobin. The red cell count was performed with an Electrical Cell Scope (Ljungberg AB red cell counter). After taking the blood samples the embryos were freed from the membranes and weighed.

Since the purpose of the study was only to investigate the erythropoietic behaviour, relative values were considered satisfactory.

In order to ascertain the chicks in the early posthatching period respond to variations in oxygen tension, chicks aged 2, 3, 13 and 29 days were exposed hours daily during 6–7 days to a low barometric pressure (400–470 mm Hg). The hematocrit was determined at the end of the exposure period.

A series of experiments was conducted in order to see whether exposure to increased oxygen tension (40 % O_2 in N_2) would likewise affect the hematocrit level of chicks. Six-day-old chicks were divided into 2 groups with 7 chicken in each and reared in 2 glass containers ventilated

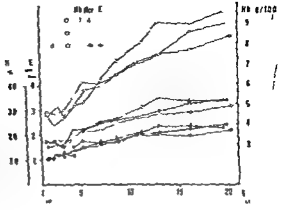

Fig 2 Hematological values (hemoglobin hematocrit erythrocytes) of chick embryos incubated in different oxygen tensions

with air and with a 40 % O gas mixture respectively for 9 days The hematocrits were determined from cubital vein blood

Statistical evaluation Because of the great variability in the development of chick embryos the hematological values show considerable variations The ordinary Student s test for evaluation of the statistical significance of differences is therefore not quite adequate The Wilcoxon test which is recommended for data with a large variability and indefinite distribution of the values (Geigy AB 1961) was used instead

Results

Although the number of experiments is small the experiments with exposure to low pressure showed quite clearly that the 2—7 [illegible] days old chick reacts with increased hematocrit to the hypoxic stimulus The observation is consistent with the results obtained with older chicks Since the intensity of reaction does not interest us in this connection we saw no reason to continue these experiments Fig 1 shows the results

It is seen furthermore that there seems to prevail a slight tendency of the chick to reduce the hematocrit level as a response to increased oxygen tension (Fig 1 rhombi) However the difference does not reach statistical significance the probability level being approximately 0 1

Effect of oxygen tension upon hematological values in chick embryo

Because variations in ambient oxygen tension tend to affect the growth rate of the embryo a comparison cannot be based on equal age of incubation Therefore the embryos were divided into groups of equal weight and the graphs were constructed to show the hematological values as a function of embryo weight Fig 2 shows variations in the erythrocyte count (mill/µl) hematocrit (per cent packed red cells) and Hb (g/100 ml blood) as a function of embryo weight (11 weight groups) During the relatively early developmental period i e before the 14th incubation day and an embryo weight of less than 7 g none of these variables seems to be affected by the difference in oxygen tension although they increase with increasing embryonic weight In the heavier (and older) embryos a tendency of the low oxygen group values to exceed those of the 40 % oxygen group becomes clearly visible However it is a well known fact that chick embryos show very large variations whatever is being measured

TABLE I

	15 O	21 O	40 O
n	13	9	17
Weight g	1 60±0 [illegible]	1 99±0 12	1 89±0 11[illegible]
Hb g/100 blood	4 2 ±0 24	4 6 ±0 25	4 5 ±0 22
Hcr	16 8 ±1 1	15 7 ±0 89	17 4 ±0 93
E mill/mm³	1 08±0 12	1 05±0 10[illegible]	1 05±0 06[illegible]
Age range days	9—10	9—11	9—11
n	12	13	23
Weight	3 21±0 11	3 01±0 13	3 03±0 0[illegible]
Hb	4 3 ±0 34	4 6 ±0 35	4 8 ±0 22
Hcr	14 8 ±0 84	15 3 ±1 3	17 1 ±0 83
E	1 20±0 091	1 29±0 11	1 18±0 0[illegible]0
Age range	11—12	10—12	10—12
n	13	17	3[illegible]
Weight	5 10±0 18	4 99±0 21	5 31±0 15
Hb	5 1 ±0 35	5 6 ±0 31	5 3 ±0 17
Hcr	20 5 ±1 41	22 1 ±1 44	19 1 ±0 [illegible]4
E	1 43±0 15	1 55±0 10	1 43±0 043
Age range	12—13	12—14	11—14
[illegible]	8	21	24
Weight	8 22±0 27	8 48±0 20	8 31±0 [illegible]
Hb	6 7 ±0 27	6 3 ±0 25	6 4 ±0 23
Hcr	2[illegible] 7 ±1 03	25 5 ±0 9[illegible]	23 8 ±1 00
E	1 72±0 081	1 65±0 083	1 [illegible]8±0 0[illegible]4
Age range	14—15	13—16	13 [illegible]
n	15	13	25
Weight	11 7 ±0 20	11 4 ±0 30	11 [illegible] ±0 21
Hb	8 4 ±0 24	7 1 ±0 3[illegible]	7 [illegible] ±0 2[illegible]
Hcr	31 8 ±0 83	[illegible]7 2 ±1 4[illegible]	[illegible]6 9 ±0 [illegible]0
E	2 14±0 071	2 01±0 14	1 95±0 079
Age range	1[illegible]—18	15—18	14—17
n	11	7	21
Weight	14 6 ±0 41	15 1 ±0 37	14 3 ±0 [illegible]1
Hb	[illegible] ±0 31	8 0 ±0 45	7 5 ±0 21
Hcr	33 5 ±0 60	31 1 ±2 1	28 1 ±0 [illegible]8
E	2 34±0 1[illegible]	2 17±0 1[illegible]	1 93±0 039
Age range	16—18	15—18	15—18
n	12	11	[illegible]
Weight	18 3 ±0 5[illegible]	18 0 ±0 64	18 9 ±0 42
Hb	9 3 ±0 2[illegible]	8 9 ±0 31	8 2 ±0 [illegible]
Hcr	33 9 ±1 1	33 5 ±1 21	30 2 ±0 78
E	2 3[illegible]±0 091	[illegible] 27±0 097	[illegible] 11±0 0[illegible]7
Age range	1[illegible]—18	1[illegible]—18	16—19

Mean ± S. E. of the mean

Statistical evaluation (Wilcoxon test) of difference between values obtained at 40 % O_2 and

15 O P		21 % O P		15 % and 21 % combined P	
		Weight range 1 00—2 49 g			
>0 1	—	>0 1	—	>0 1	—[1]
>0 1	—	>0 1	—	>0 1	—
>0 1	—	>0 1	—	>0 1	—
		Weight range 2 50—3 99 g			
>0 1	—	>0 1	—	0 1	—
=0 1	—	>0 1	—	0 1>P>0 05	—
>0 1	—	>0 1	—	0 1	—
		Weight range 4 0—6 99 g			
>0 1	—	0 1	—	0 1	—
>0 1	—	0 1>P>0 05	—	0 1>P 0 05	—
>0 1	—	>0 1	—	>0 1	—
		Weight range 7 0—9 9 g			
>0 1	—	0 1	—	0 1	—
<0 01	<	>0 1	—	0 1	—
>0 1	—	>0 1	—	>0 1	—
		Weight range 10 0—12 9 g			
0 01		0 1	—	0 1 P 0 05	—
<0 01		0 1	—	0 05	
=0 1	—	0 1	—	>0 1	—
		Weight range 13 0—15 9 g			
0 01		0 1	—	0 01	
<0 01		0 1	—	<0 01	<
<0 01		0 1	—	0 01	
		Weight range 16 0—20 9 g			
0 05		>0 1	—	0 0	
0 01		0 05		0 05	×
0 1	—	0 1	—	0 1	—

Significance

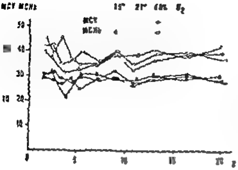

Fig 3 Mean corpuscular volume and mean corpuscular hemoglobin content as a function of embryo weight in embryos incubated at different oxygen tensions

In Table I the hematological values and the statistical evaluation of results (Wilcoxon test) are shown As may be seen and as suggested by the curves in Fig 2 there is no significant difference between any of the hematological values in embryos developed at 40 % O_2 as compared with those developed either in air or in 15 % O_2 if the embryo weight is less than 7 g In the weight group 7—9 9 g the hematocrit of the embryos incubated in 15 % O_2 exceeds significantly that of the 40 % O_2 controls and in the next heavier group both Hb and hematocrit but not the erythrocytes show a similar difference In the weight group 13 0—15 9 g the erythrocyte count is also significantly higher in the 15 % O_2 embryos than in the 40 % O_2 group On the whole however the difference in the erythrocyte count is so small that attaining of statistical significance would require a very large number of experiments The difference between the hemoglobin values referring to the 15 % O_2 group and the group of embryos incubated in air is also of some interest In the heaviest embryos there is no difference At embryo weight groups from 10 0 to 15 9 g the differences between air and 15 % values again are significant At smaller embryonic weights however there is a reversal of the values The highest Hb values are in the 21 or 40 % O_2 group and the lowest in the 15 % O_2 group However these differences do not quite attain statistical significance in any of the weight groups Furthermore it is to be noted that the mean weight of the smallest embryos is less in the 15 % group than in the 21 % group which might partly explain the smaller Hb concentration

The sensitivity of the hematocrit for differences in ambient oxygen tension makes it necessary to compare the mean corpuscular volume (MCV) in the experimental groups in order to rule out the possibility that the observed reactions were actually dependent upon variations in volume of the cells Likewise the mean corpuscular Hb (MCHb) was calculated in order to see whether the increased Hb concentration is due mainly to increased cellular Hb or to increased number of cells with equal Hb content The results of these calculations are seen in Fig 3 It is obvious that the variations in the curves are not systematically dependent upon ambient oxygen concentration Except for an initial slight drop in the mean corpuscular Hb common for all 3 experimental groups the curves both those representing MCHb and those of MCV run practically horizontally

Discussion

If the general trend of the variations in Hb concentration erythrocyte count and hematocrit during embryonic development is compared with those found in previous studies

the correspondence is quite satisfactory (for ref Romanoff pp 594—596 1960) Studies concerning the effect of ambient oxygen concentration on hematological values in the chick embryo are but few and they deal mostly with embryos incubated for a few days only The paper of Flemister and Cunningham (1940) concerning the effect of increased atmospheric pressure upon development of the vasculature and hematological values in chick embryos seems to be contradictory to the present findings in so far that they found very much lower (about 50 per cent) Hb content and erythrocyte count in ▮ day old embryos developed at a pressure of 40 pounds than in embryos incubated at atmospheric pressure However the Hb concentrations in the control embryos aged ▮ and 10 days were higher than in any of our embryos of the same age or those mentioned in other papers (Zorn and Dalton 1937 Pilipenko 1957 Barnes and Jensen 1959) On the other hand, the "decreased values in the high pressure embryos exceeded the normal values for chick embryo Hb usually found at that age

If the present experiments are compared with a similar study made by the senior author a few years ago (Astola *et al* 1959) there is complete agreement as far as comparable data exist Thus the independence of the total Hb content of variations in the ambient oxygen concentration in the early chick embryo up to the age of 9 days corresponds to the similar independence of the relative Hb values erythrocyte count and hematocrit up to age of some 14—16 days in this study The difference between the hemoglobin concentration of the embryos developing in 15 % O and those developing in 40 % O_2 does not seem to appear until in the last week before hatching Our working hypothesis that the sensitivity to oxygen lack develops during the ontogeny seems thus warranted However the question arises whether the phase of embryonic development at which the reactivity of the erythron to hypoxia begins to be manifested depends upon the degree of hypoxia and the metabolic need of oxygen Thus one could assume that during the later stages of embryonic life a relative lack of oxygen exists even in the embryos incubated in air but that no such disproportion between oxygen need and supply exists in the early chick embryo with an HbO_2 dissociation curve shifted to the left (Hall 1934) even at lower ambient oxygen concentrations This assumption however has very little probability Firstly O_2 concentrations lower than 15 already increase the mortality in the early stages quite considerably Secondly It is not likely that a more severe hypoxia would exert a stimulatory effect because even the 15 % O concentration seems rather to depress than to stimulate the erythron of the young embryos weighing less than 7 g and being less than 14 days of age

If the age of 15 days as suggested by the present data would be accepted as a limit of reactivity of the erythron to hypoxia in the developing chick embryo a question poses itself as to what else is happening in the organism at that phase Considering the blood forming tissues it is at about this time or slightly earlier 12—14 days that blood formation begins in the bone marrow ▮ would therefore be tempting to conclude that the reactivity of the erythron is mainly dependent on the bone marrow Unfortunately this conclusion cannot be made because the 15th day of embryonic development seems to represent a critical point in many other respects too Thus for instance the synthesis of some polyamines e g spermine begin at that date (Raina 1963 and the stability of serum proteins increases suddenly at the 16th day (Schechtman 1952 Although the weight curves of different organs show mostl a steady gain of weight e g lungs heart, liver brain) during the entire embryonic period some endocrines the hypophysis and the testes reach their maximum embryonic weight already at the 16th day of

incubation and the hypophysis becomes functionally active at that date (Case 1952). Likewise the maximum weight of the mesonephros is being attained approximately at the 15th day after which it begins to regress (data compiled by Romanoff 1960 pp 1149—1151) More direct experiments are therefore needed before definite conclusions are possible Examination of bone marrows for hematopoetic foci and electrophoretic determinations of the blood proteins in embryos incubated at different oxygen tensions are the nearest targets of our investigations

As mentioned in the introduction the question of erythropoietins is closely linked to the reactivity of the erythron against hypoxia One may ask whether the unresponsiveness of the early chick embryo is due to inability to form erythropoietins or to inability to respond to erythropoietins

This we intend to study injecting chick embryos with cobalt and with erythropoietin containing extracts in different phases of embryonic development

With the technical assistance of T Haapiainen A Keskinen P L Länsipuro M Murismaa and L Seppala

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Acta physiol scand 1965 63 487—494

From the Laboratoire de Physiologie Faculté de Médecine Toulouse France

Central Control of Static and Dynamic Sensitivities of Muscle Spindle Primary Endings

By

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Abstract

Appelberg B and F Emonet Denand *Central control of static and dynamic sensitivities of muscle spindle primary endings* Acta physiol scand 1965 63 487—494 — In cats under light Nembutal/urethane anesthesia electrical stimulation within a restricted area in the mesencephalon caused a strong increase of the dynamic sensitivity of extensor as well as flexor muscle spindles Static effects were usually weak from this region and sometimes inhibition of static activity was evoked simultaneously with dynamic activation From neighbouring regions augmentation or inhibition of static sensitivity was obtained without effects on the dynamic sensitivity By using small muscles and recording electromyograms it could be shown that the effects on the spindles were elicited without simultaneous extrafusal activation The conclusion is reached that central stimulation in appropriate regions may in a rather selective way activate different populations of gamma efferent fibres having either a dynamic or a static effect on the spindles The results are discussed in relation to recent investigations of the effect of single gamma fibre stimulation on spindle sensitivity

The discharge frequency of primary afferents from muscle spindles is dependent upon muscle length and upon the activity in motor fibres to the spindles During steady fusimotor discharge or in de efferented preparations the primary discharge is proportional to the maintained length of the muscle (the static response) During the phase of extending a muscle the discharge of the spindle primary afferents shows a phasic behaviour The size of this phasic burst of impulses (the dynamic response) is related to the velocity of the extension The static as well as the dynamic sensitivity of the primary endings may be increased by intrafusal muscle contraction

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[2] The term dynamic response was used by Jansen and Matthews (1962) to mean the difference in frequency between a point at the end of the period of stretching and a point 0 5 sec later Crowe and Matthews (1964a) suggested the term dynamic index to replace the initial one In the present context the term dynamic response is used to mean the burst of impulses seen during the period of extending the muscle

Jansen and Matthews (1961, published in full 1962) suggested that the efferent innervation of muscle spindles is so organized that the static and dynamic sensitivities can be independently controlled. These authors found that changes in fusimotor activation of spindles induced by ventral root cutting, reflex stimulation or spontaneously occurring in decerebrate preparations caused unrelated variations in static and dynamic sensitivities of individual spindles. Later experiments by Matthews (1962) indicated the existence of two groups of fusimotor fibres, the one increasing static sensitivity and diminishing the "dynamic index", the other augmenting mainly the "dynamic index" and acting only weakly on the static response.

Lately experiments have been carried out to study the action of a single gamma fibre on several spindles (Crowe and Matthews 1963, 1964 a, b; Bessou, Laporte and Pagès, personal communication). This work has shown that stimulation of a given single gamma fibre causes an increase of either static or dynamic sensitivity in all spindles to which the fibre is distributed; this seems to prove the existence of a peripheral organization for modifying selectively dynamic and static sensitivities. It should, however, be noted that according to the last mentioned authors fibres with effects intermediate between static and dynamic are found.

During experiments with electrical stimulation in the region of the red nucleus observations were made which seemed to indicate that stimulation of adjacent but separate regions act differently on the static and dynamic sensitivities of spindles in the gastrocnemius muscle. In experiments designed to investigate this phenomenon it could be shown that an increase of gastrocnemius spindle dynamic sensitivity can be obtained from a restricted region in the mesencephalon (cf. brief communication by Appelberg, 1963).

The experiments to be described were undertaken in an attempt to determine whether the central nervous system may selectively influence the different groups of fusimotor fibres found in the periphery. Fusimotor fibres to some muscles also include a group of slow-conducting alpha fibres (Bessou, Emonet-Denand and Laporte 1963; Emonet-Denand 1963). With regard to this fact, small muscles, one extensor and one flexor, were used, since in such muscles any activation of alpha fibres could more easily be detected (for further description and references see Results). The results obtained show that central stimulation in appropriate regions can activate selectively gamma efferents which have mainly a dynamic or a static effect on the spindles.

Methods

41 cats were used for the experiments. In the beginning of the experimental series pure Nembutal was used as an anesthetic. It soon became evident that the repeated administration of Nembutal during extended experiments too much depressed the central nervous system. By using a mixture of Nembutal ([illegible] mg/kg) and urethane ([illegible] mg/kg) the total dose of Nembutal was considerably reduced and the animals remained in a more favourable condition. The anesthetic level was important for the elicitation of dynamic effects. The most successful experiments were carried out on animals having some tone, especially in the fore limbs and in neck and cheek muscles and possessing a clear-cut flexor reflex upon pinching a paw.

The operative procedure included exposure of the lumbar and sacral spinal cord after laminectomy. The right dorsal and ventral roots L[illegible] to S[illegible] were usually cut extradurally to avoid movements caused by central stimulation in the leg and tail. On the left side all ventral roots were left intact while the dorsal roots L[illegible] to S[illegible] were cut and used for recording. The left hind limb was denervated as completely as possible except for the muscle being studied. The muscles used were the medial or lateral gastrocnemius, soleus nerve cut, or the peroneus tertius (flexor

of the foot, extensor and abductor of the fifth digit) or the flexor longus digitorum (extensor of the foot). The nerve to the muscle under study and the muscle itself were exposed and covered by a pool of liquid paraffin kept at body temperature. The leg was immobilized by rigidly clamping the upper and lower ends of the tibia. The tendon of the muscle was connected to a myograph via the pulling device previously described (Appelberg 1962b).

The activity of single muscle spindle afferents was recorded from thin dorsal root filaments. The spindle character of an ending was ascertained by observing the pause in its discharge during a muscle twitch. Only fibres having the characteristics of primary endings, i.e. conduction velocity well above 75 m/sec and showing a dynamic component in their response to extension, were used. The response of the spindle to extension of the muscle was recorded under control conditions and during electrical stimulation in the brain. For reasons which will be mentioned under Results, electromyographic recording was chosen as the standard method for revealing activation of alpha fibres to the muscle studied. This was accomplished with the aid of two Ringer-soaked wick electrodes in contact with the upper and lower parts of the belly of the muscle.

The lead from the afferent fibre, the electromyographic recording and the myograph output (recording the tension developed in the muscle during extension) were displayed on two double beam oscilloscopes and photographed on film moving at 50 mm/sec. After the experiments instantaneous frequency curves were made by measuring spike intervals on magnified traces of the film and constructing graphs with the reciprocal values of these intervals. In a few experiments at the end of the series an instantaneous frequency meter was used instead.

The brain was stimulated with the aid of a concentric stimulating electrode guided according to the Horsley Clarke stereotaxic technique. Stimulation consisted of trains of square wave impulses at a frequency of 300 imp/sec and with a pulse duration of 1 msec. The duration of individual stimulations was kept as short as possible, usually about five seconds. In addition one or two minutes were allowed to pass between successive stimulation periods as rebound diminution of central excitability was often noted.

Stimulation was usually carried out on the left side, i.e. ipsilaterally to the recording side. Histological verification of stimulating electrode sites was not made in the present series of experiments.

Results

The first experiments constituted a directed continuation of the work previously reported (Appelberg 1963). It could be confirmed that electrical stimulation contralaterally in the brain in a region stereotaxically localized to be within the red nucleus caused a strong increase in gastrocnemius spindle dynamic sensitivity. Ipsilateral stimulation at a level probably slightly caudally to the red nucleus caused the same type of effect and this stimulating site was used during the rest of the series. The stimulation causing an increase of dynamic sensitivity of spindles often also modified their static response. Such static effects were, however, completely unrelated to the dynamic effect and varied for extensor spindles between fairly strong excitation to clear-cut inhibition (Fig. 1 in Appelberg 1963 demonstrated an occasion where the static effect was practically zero but where the dynamic sensitivity of the spindle was strongly increased).

Dynamic activation of muscle spindles may according to present knowledge be accomplished in two different ways, either by activation of dynamic gamma fibres (see above) or via slow conducting alpha fibres having intrafusal connections. The last mentioned type of fibres have been shown to exist in the deep lumbrical muscles of the cat (Bessou *et al.* 1963 a, Emonet-Denand 1963) and the possibility of their presence in other muscles cannot be excluded. The stimulation of such fibres cause an increase of spindle dynamic sensitivity (Bessou *et al.* 1963 b). These slow alpha motor units are further known to cause only small muscle action potentials, they have weak twitch contractions (ranging between 0.1 and 0.9 g) and their tetanic tension is usually less than 3 g (Steg 19[illegible], 1964, Emonet-Denand 1963, Appelberg, Emonet-Denand to be published). With regard to the very small changes in tension produced by these fibres it

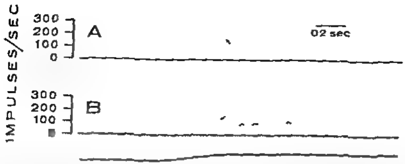

Fig 1 Recording from extensor spindle afferent (flexor longus digitorum) Each dot represents one spike and in the vertical plane also indicates the instantaneous frequency of this spike compared to the preceeding one (see frequency scale to the left) A is the control record ng B was obtained while stimulating the dynamic excitatory region During both recordings the muscle was extended linearly 4 mm from an initially practically unloaded position The period of extension is indicated by oblique part of the line below the recordings

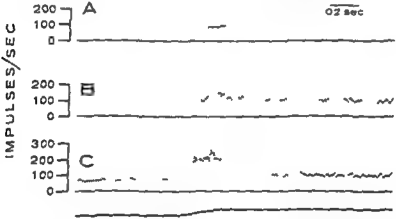

Fig 2 Recording from flexor spindle afferent (peroneus tertius) A is the control record ng B was obtained while stimulating the mesencephalic reticular formation and C during stimulation within the dynamic excitatory region Muscle extension in all recordings 4 mm from unloaded position (indicated by oblique part of line below recordings)

was considered advantageous to use electromyograpl c recording as a ensit ve check of alpl a activation instead of conventional tension recording Further a small muscle was chosen to allow even small electrical events in the interior of the muscle to be recorded on its surface

In experiments carried out on the flexor longus digitorum muscle it could be shown that dynamic activation of spindles was obtained without any electromyographic changes being recorded in the muscle i e without any simultaneous alpha activation It should be noted here that in spite of an existing tone in many muscles especially in the anterior part of the preparation spontaneous alpha activity in the muscles used was but rarely observed Fig 1 furnishes an example of dynamic activation of a spindle in

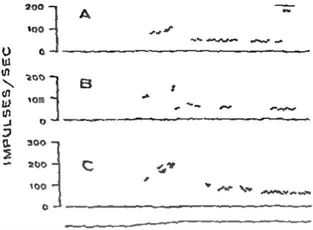

Fig 3 Recording from spindle afferent from peroneus tertius. A and B were both obtained as controls but at different stages of the experiment Note spontaneous change mainly in dynamic sensitivity C was recorded during electrical stimulation in the dynamic excitatory region and shows a strong increase in dynamic sensitivity with practically no static effect In all recordings the muscle was extended 5 mm from an initially practically unloaded position.

the flexor longus digitorum Central stimulation in the dynamic region moderately increased the static discharge of this spindle as seen when comparing recordings A and B before beginning of stretch or at the end of the recordings when the spindle has adapted to the new length of the muscle The change in behaviour of the spindle during the phase of extension was however much more important. During stimulation in B the spindle thus starts to react to the extension earlier than in A and then continuously increases its rate of firing throughout the phase of extension

The dynamic sensitivity of muscle spindles in the peroneus tertius muscle could be readily increased by stimulation in the same region as for extensor spindles Here also the dynamic effect could regularly be obtained without concomitant alpha activation The simultaneous effects on the static response of flexor spindles were always more or less excitatory It was considered valuable to compare in the same spindle a centrally evoked static effect with one of the dynamic augmentatory type Such a comparison is demonstrated in Fig 2 where A is a control B was obtained while stimulating the formatio reticularis mesencephali and C was recorded during stimulation in the usual region deeper in the brain stem The activation of the spindle in B concerns only the static discharge and the dynamic burst is consequently practically absent In C on the other hand the spindle is statically activated to about the same degree before stretch but also responds vigorously during the phase of extension producing a dynamic burst considerably bigger than in A and B The frequency then rapidly falls to a level close to that in B

An interesting observation which was frequently made is illustrated by the difference between the recordings shown in Fig 3 A and B These were both obtained as controls without any simultaneous central stimulation but at different stages of the experiment The recordings demonstrate a spontaneous increase in the dynamic sensitivity of this spindle Similar spontaneous changes of static sensitivity were also encountered Fig 3 C

again shows the effect of central stimulation, which in this case caused a considerably stronger dynamic activation of the spindle than was spontaneously evoked. Occasionally such a strong spontaneous augmentation of the dynamic sensitivity occurred that the central stimulation was more or less without effect. Fig. 3 C also gives a demonstration of a fairly slow decrease of frequency occurring after the initial rapid drop of the discharge at completion of stretch. Crowe and Matthews (1964 a) noted this slow component of the dynamic response and attributed it to a slow decay of tension in the intrafusal muscle underlying the dynamic receptors.

The above described dynamic effect, as well as inhibitory and facilitatory static effects occasionally observed, were thus, as previously mentioned, obtained without action potentials being recorded from the muscle. The spindle control as it has been presented above is therefore exerted via purely fusimotor pathways, i.e. via the gamma motor system. It was, however, regularly observed for extensor as well as for flexor spindles that an augmentation of the intensity of the central stimulus in the dynamic area increased the dynamic effect on the spindles but simultaneously brought in small spikes in the electromyogram. These muscle action potentials were never associated with visible contractions of the muscle and differed considerably in size from big spikes occasionally seen occurring simultaneously with twitches in the muscle. It is possible, therefore, that the electromyogram evoked by stronger stimulation in the dynamic region, and being associated with an increased dynamic effect on the spindles, was caused by activity in slow alpha motor units. Attempts to evoke this type of alpha activation from nearby regions without dynamic spindle effects being elicited were not successful. Obviously the present experiments do not reveal whether the increased dynamic effect on the spindles in this case was caused by intrafusal branches of slow conducting alpha fibres being activated, or was simply due to a more complete stimulation of the dynamic gamma motoneurones.

Difficulties in obtaining the dynamic effect could almost always be referred to the anesthesia being too deep or, occasionally, to the stimulating electrode being wrongly placed. It was frequently observed that even small additional injections of the anesthetic completely prevented the transmission of the dynamic effect while the static effects were still obtained. Under favourable experimental conditions the elicitation of the dynamic effect seemed to be independent of the peripheral end organ chosen. Even if quantitative differences in the reaction of individual spindles were seen, the results described are typical for all spindles investigated in flexor as well as in extensor muscles.

Discussion

The results of the present investigation demonstrate the existence of separate regions within the central nervous system the stimulation of which causes a setting of muscle spindle sensitivity in either dynamic or static direction. From the fact that electromyographic recording at the surface of the small muscles used detected no signs of extrafusal activation during the periods of central stimulation, it must be concluded that the effects studied were transmitted via purely fusimotor pathways, i.e. via the gamma motor system. The increase in dynamic activation of the spindles upon augmenting the central stimulation may have been caused by activation of slow-conducting alpha fibres having intrafusal connections, but may alternatively reflect only a stronger gamma activation.

A concept of the fusimotor system as being strictly separable into two functional fibre groups having either purely static or purely dynamic action on the spindles is probably simplified Bessou Laporte and Pages (personal communication) have observed that some gamma fibres cause effects intermediary between dynamic and static With this in mind it is not surprising to find that central stimulation is not always capable of producing completely selective static or dynamic effects It is becoming more and more evident that the region of the red nucleus is an important area for muscle spindle control (see also Appelberg 1962 a b Appelberg and Kosary 1963) It seems likely that from such an area the spindles may by appropriate activation of their motor fibres be set at any desired combination of static and dynamic sensitivity During the present experiments varying static effects were observed to occur simultaneously with the dynamic activation. This was probably due to the stimulation activating mainly a region controlling dynamic gamma motoneurones but to a certain extent spreading also to neighbouring regions so as to yield some static or mixed effects

The observation that the central nervous system left to itself under fairly light anaesthesia may spontaneously alter in an independent way dynamic or static properties of the spindles is interesting Similar observations were reported by Jansen and Matthews (1962) on decerebrate cats The functional significance of independent central control of muscle spindle static and dynamic sensitivity seems to be emphasized by these findings

The importance of the depth of anesthesia for the elicitation of various effects on the gamma system from the region of the red nucleus is remarkable In the deeply anesthetized animal the spindles behave as de efferented and even strong central stimulation does not alter their regular discharge The first signs of connection between the brain and the periphery are continuous variations in the static discharge of the spindles In this stage decrease of static sensitivity due to gamma inhibition is the only effect caused by rubral stimulation In a more active preparation facilitation of static sensitivity is evoked from parts of the red nucleus (cf Appelberg and Kosary 1963) The dynamic effects described in the present work seem to be even more sensitive to the influence of the anesthetic used It seems likely that these differences in susceptibility to anesthesia reflect differences either in number of neurones in the pathways concerned or in the qualities of different pools of neurones in the stimulated area

The fact that ipsilateral stimulation was employed in the present series of experiments does not exclude the participation of the red nucleus in the effects observed It is believed that the stimulation affected either the already crossed rubrospinal tract or fibres from the cerebellum destined for the red nucleus This suggestion is supported by the observation in preliminary experiments that contralateral stimulation stereotaxically localized to the red nucleus produced identical effects Also dynamic effects were obtained by histologically localized rubral stimulation by Appelberg (1962 b) These effects were then described as resulting from mechanical events within the spindle and thus as being secondary to the static effects simultaneously evoked It may now be supposed that the results were due to the dynamic area having been unwittingly stimulated Further work with appropriate histological localization of stimulating points will however be needed to elucidate this point

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From the Departments of Thoracic Surgery Physiology and Clinical Physiology University of Goteborg Sweden

Coronary Collateral Blood Flow in Relation to the Mass of Ischemic Myocardium, Studied with Krypton[85]

By

Borje Johansson Erland Linder and Torsten Seeman[1]

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Abstract

Johansson [redacted] E Linder and T Seeman *Coronary collateral blood flow in relation to the mass of ischemic myocardium studied with Krypton* Acta physiol scand 1964 63 495—504 — Blood flow in the ischemic region during acute coronary occlusion in anesthetized open chest dogs was studied by external recording of wash out curves after intracoronary administration of Kr[85] Flow values were satisfactorily well reproducible in 29 double determinations Collateral blood flow ranged from 6 to 44 ml/min 100 g tissue The magnitude of flow was inversely related to the weight of the ischemic region in 14 of 20 dogs In 6 dogs with the lowest flow values this correlation was only slightly indicated Arterial occlusion did not seem to affect flow in adjacent nonischemic myocardium The potential advantages of the present technique for the study of collateral blood flow are discussed in comparison with other methods

An analysis of wash out curves recorded externally during elimination of Krypton[85] from the myocardium in open chest dogs subjected to acute coronary occlusions was presented in a previous paper as a method of measuring coronary collateral blood flow CCBF (Johansson Linder and Seeman 1964) It is shown in the present study that double determinations of CCBF made with this technique under stable cardiovascular conditions give results which are satisfactorily well reproducible Further the dependence of the collateral blood flow on the extension of the ischemic region has been studied by alternative occlusions of the anterior descending coronary artery and its main branches

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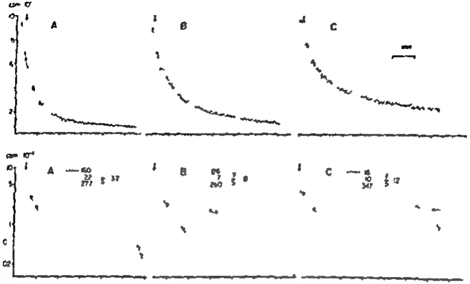


Fig 1 Above Wash out curves from one experiment with occlusion of smaller sidebranch (A) larger sidebranch (B) and main trunk (C) of left anterior descending artery
Below Corresponding semilogarithmic plottings resolved into a slow phase representing coronary collateral blood flow (CCBF) and a fast phase representing blood flow in normally perfused myocardium Calculated values for CCBF demonstrate its dependence on the size of the ischemic region The quotient F/S is obtained from the zero time activity values of fast and slow phase and is an expression of normally perfused versus ischemic myocardial mass The extended ischemic mass from A to C is reflected in these quotients The terminal rapid wash out of indicator corresponds to reactive hyperemia after unclamping

Methods

The results presented in this report were obtained in experiments on 20 dogs The experimental procedure has been described fully in the previous paper (Johansson *et al* 1961) to which the reader is referred for methodological details not given below In principle the animals were anesthetized i v with pentobarbital curarized and artificially ventilated with open system The heart was exposed by a left thoracotomy A fine polyethylene catheter to be used for close-arterial injections of Kr[85] solution was sutured into the left anterior descending coronary artery Sections of this vessel distal and/or proximal to the site of injection were dissected free and placed in snares for subsequent occlusion The elimination of Kr[85] from the myocardium after close arterial administration was observed by external recording and the wash out curves obtained were analysed as described in the previous publication
Arterial blood pressure and ECG were recorded continuously Repeated determination of cardiac output were done during the course of the experiments using the Krypton technique described by Cornell Braunwald and Brockenbrough (1961) To keep the general cardiovascular state as constant as possible arterial blood pressure was adjusted in many experiments by means of a pressure bottle connected to one of the femoral arteries and heart rate was controlled by electrical stimulation of the ventricles after blocking the A V transmission by injection of formaline or alcohol into the septum (Cutman *et al* 1959)
The approximate size of the myocardial regions subjected to ischemia by the different coronary artery occlusions was determined at the end of each experiment These estimations were done on the basis of the gross anatomy of the vessels and of the coloring obtained by injection of dye (Kenton fast green) the different branches of the anterior descending coronary artery The respective portions of the myocardium were cut out and weighed Also the total weight of the heart and of the left ventricle were noted

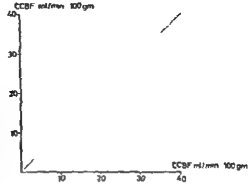


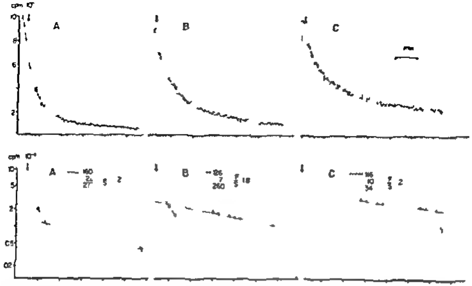
Fig 2 Paired values from 29 double determinations of CCBF in 16 expts Deviations from line of identity less than 15 per cent

Results

The wash out recordings from one experiment (Fig 1) are shown to illustrate the myocardial clearance of i a administered Kr[85] when arterial branches of different caliber were occluded Curve A was obtained when a smaller side branch of the left anterior descending artery was occluded instantaneously at the arrow on the curve Curve B was recorded after occlusion of the larger branch from the same division of the main trunk Occlusion of the latter proximal to the bifurcation resulted in the decay curve in C Krypton[85] was injected in all three recordings through a thin polyethylene catheter sutured into the artery some cm proximal to the occlusion site in C When plotted semilogarithmically as described in a previous communication (Johansson *et al* 1964) and shown below the recordings in Fig 1 these curves can be resolved into two distinct components By using the rate constant of the slow component it is possible to calculate the amount of blood flowing into the ischemic region *via* intercoronary anastomoses and possibly from other sources This flow will be referred to as coronary collateral blood flow CCBF Values for CCBF calculated from the actual recordings were in A 22 in B 17 and in C 10 ml/min × 100 g muscle

The fast component (F) in the plottings is deduced by subtracting the ordinate values of the slow component from the composite curve and is considered to represent flow in the normally perfused muscle Calculated flow rates from these components in the actual experiment were of the same order during the inscriptions of curves B and C amounting to 126 and 116 ml min 100 g tissue respectively Furthermore they correlated well with a value of 124 ml min 100 g for normal coronary blood flow obtained before the recordings in Fig 1 The significantly higher value arrived at in A despite same heart rate and systemic blood pressure prevailed throughout all 3 recordings was probably due to the reactive hyperemia elicited by a previous occlusion since at A Kr[85] was injected within one minute after release of this occlusion According to Coffman and Gregg 1960) the reactive hyperemia ensuing extended periods of temporary myocardial ischemia usually lasts 2 min or longer

The ordinate values of fast and slow components at the moment of arterial occlusion are given as a quotient F/S in each plotting in Fig 1 According to the analysis recently presented (Johansson *et al* 1964) this quotient should reflect the size of the normally perfused versus the ischemic part of the myocardial region saturated with



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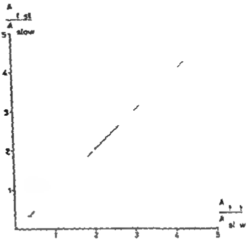

Fig 3 Calculated quotients of zero time activity values of fast and slow phase in 24 double determinations in 15 expts

and Gregg (1960) of the magnitude of the hyperemic flow after periods of coronary artery occlusion of increasing length the flow values obtained with the present desaturation technique probably correspond to peak flow after the prolonged ischemia induced in the present study Calculated values for reactive hyperemic flow in Fig 1 are in the same range in A and B but considerably higher in C after longstanding ischemia in a large myocardial region However no consistent correlation between the reactive hyperemic flow and the size of the ischemic region has been found in the present series of experiments

A rapid wash out of residual indicator after release of an occlusion has been taken as a criterion of nonrestricted arterial inflow and undisturbed clearance of Kr^{85}

The reproducibility of values for coronary collateral blood flow obtained with the present technique was studied in a series of repeated identical and mostly consecutive occlusions in 16 expts as illustrated in Fig 2 The paired values differed by less than 10 per cent in 24 double determinations and by 10—15 per cent in the remaining 5 In some of these experiments arterial occlusion was made proximal to the injection site In these cases the slow phase appeared very soon in the wash out curves (Johansson *et al*) CCBF ranged from 6 to 36 ml/min 100 g ischemic muscle in the series of double determinations

In the present study determination of normal blood flow has been made before and after each occlusion period Values calculated from these monoexponential curves correlated well with those derived from the fast components in the biphasic recordings during occlusion in an appreciable number of determinations Some experimental evidence has indicated that occlusion of an arterial branch may affect flow in neighbour branches (West Kobayashi and Anderson 1962 Guzman Swenson and Jones 1962) To study this problem further a separate experiment was designed and will be described more in detail The results are reported in Table I

Catheter for the Krypton injections was positioned in the left anterior descending artery about 2 cm from its origin and some cm proximal to its ramification into a somewhat larger left branch and a right branch Snares were placed around both branches close to the bifurcation Stable A—V block was induced and the heart

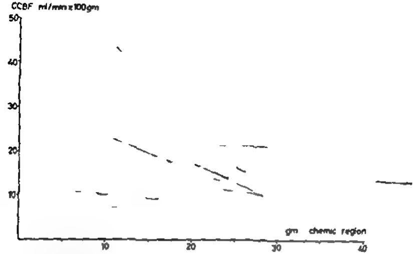

Fig 4 CCBF in relation to the estimated weight of the acutely ischemic muscle Values in each dog are interconnected

was stimulated artificially Variations in the systematic blood pressure were kept at a minimum Determination of normal blood flow without occlusion and with a heart rate of 120 was performed The "right" branch was then occluded Kr^{85} was injected within 1 min thereafter and the desaturation curve recorded Calculated flow from the resulting monoexponential decay curve did not differ grossly from the previous determination of normal blood flow Occlusion was released and somewhat later the "left" branch was occluded and Kr^{85} injected 1 min later The obtained flow value was also in the same range as in the two previous determinations as well as a flow determination without occlusion repeated shortly afterwards The next step was to occlude the "right" branch after Kr^{85} injection in order to determine coronary collateral blood flow After a sufficient time interval the same determinations were performed with the "left" branch occluded The same procedures were repeated after stepwise increase of heart rate to 160 and 200 beats/min and return to 130 as seen in Table I Values for myocardial blood flow obtained from the monophasic curves with and without occlusion and those calculated from the fast components in the composite desaturation curves during arterial occlusion did not differ systematically at any heart rate The highest value of 172 ml min 100 g was obtained within 1 min after release of a previous occlusion and is thus probably influenced by the reactive hyperemia

Evidently there was no further increment in coronary blood flow when heart rate was stepped up from 160 to 200 beats min There was a fall in total coronary vascular resistance with increasing heart rate and a readjustment to control values after restoring initial heart rate

The reproducibility of the F S quotients was studied in 24 double determinations in 13 experiments as reported in Fig 3 Two of the paired values differed by more than 30 per cent

As already mentioned the weights of the muscular portions supplied by the occluded branches were estimated at the conclusion of the experiments These weights have been plotted in Fig 4 against the corresponding values for coronary collateral blood

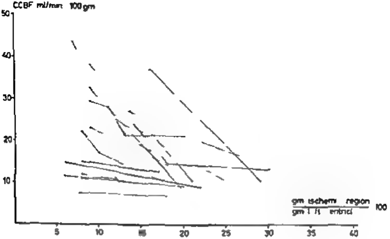

Fig 5 CCBF in relation to the size of the ischemic region expressed in per cent of left ventricular weight Same experiments as in Fig 4

flow obtained in 20 expts with occlusion of different sized arterial branches in each dog Flow values originate from the first desaturation curves with arterial occlusions inscribed under stable cardiovascular conditions in each experiment. As one might expect there is a great variability among the dogs in the magnitudes of coronary collateral blood flow and the sizes of the ischemic region None the less there is a striking correlation between flow and weight of ischemic muscle in most animals In 6 dogs with the lowest values of CCBF this flow seems little influenced by altering the mass of ischemic myocardium Three of them were markedly hypotensive which might have reduced the CCBF Despite this fact probably all six of these dogs had collateral pathways with more limited flow capacity than the others The opposite was true especially in one dog with a coronary collateral blood flow of 14 ml/min × 100 g within an ischemic muscle mass of 53 g representing 37 per cent of left ventricular weight

No values for CCBF were obtained from ischemic regions of less than 5 g In these cases no slow phase of sufficient durat on appeared at the end of the recordings before indicator activity had fallen to the background level On the other hand determinat on of CCBF was made only once in an ischemic muscle region larger than 35 g This was due to the high frequency of ventricular fibrillation resulting from occlusion of an arterial branch of that caliber

As seen in Fig 5 CCBF has been stud ed within ischem c regions ranging from 6 to 37 per cent of total left ventricular weights

Discussion

Several different methods have been used in previous experimental stud es of myocard al collateral blood flow after acute occlusions of coronary arteries

Retrograde flow from the l gated artery has been used as an indirect measure of the collateral blood supply When the anterior descending coronary artery of the dog

supplying some 50 g muscle was acutely ligated a retrograde outflow in the range of 3 ml/min was obtained (Gregg and Fisher 1963). Such measurements have been assumed to overestimate collateral blood flow due to the relatively low flow resistance of the draining cannula as compared to that of the distal coronary vascular bed. However, studies of myocardial uptake of Rb^{86} have indicated that the capillary blood flow within the acutely ischemic heart muscle is considerably greater than the retrograde flow from the same region (Levy, Imperial and Zieske 1961). They found with Rb^{86} a CCBF corresponding to 33—75 per cent of blood flow in the normally perfused myocardium (see also Chansky and Levy 1962). The discrepancy between values of CCBF obtained with the two techniques seemed to indicate that the ischemic region received additional blood supply through channels other than intercoronary anastomoses.

Myocardial uptake of deuterium oxide was used as an indicator of tissue blood flow in the experiments by Mc Lean, Hedenstrom and Rayner (1962). They found an uptake in acutely ischemic heart muscle which averaged 7 per cent of that in the normally perfused regions. The elimination of NaI^{131} after direct intramyocardial injection was recorded externally by Hollander, Madoff and Chobanian (1961, 1963). Total occlusion of the coronary artery supplying the actual region was reported to stop elimination of indicator completely.

Studies of uptake or elimination of indicator substances should be expected to reflect collateral capillary blood flow better than measurements of retrograde flow. It is evident, however, that there are considerable divergences between the results obtained with the various indicator techniques mentioned above. In the present study with Kr^{85} the values for CCBF after occlusion of the main artery agree best with those of Mc Lean and co-workers using D_2O. The fact that these two indicators are both inert and rapidly diffusing molecules probably explains this conformity of the results. The comparably high uptake Rb^{86} found in ischemic myocardium by Levy and collaborators may be at least partly explained by the relatively greater extraction of Rb at low flow rates (Nolting *et al.* 1958, Love and Burch 1959). With the method of local injection of an indicator as in the experiments by Hollander, Madoff and Chobanian (1961, 1963) catheterization of coronary arteries is avoided and also it should be possible to determine flow within certain restricted areas or even layers of the myocardium. It remains to be shown, however, that elimination of a tracer from such a local injection is simply flow dependent and is not limited by the diffusion distances.

Part of the present report has been concerned with the reproducibility of the values for CCBF obtained with Kr^{85}. The series of double determinations presented in Fig. 2 shows a difference between paired values of less than 15 per cent. Estimations of CCBF with this technique are thus satisfactorily reproducible under stable cardiovascular condition. Significantly larger alterations in CCBF have been produced by experimental changes of arterial blood pressure, hematocrit and blood viscosity (Johansson, Linder and Seeman, to be published).

As indicated by the results of alternative occlusions at different levels of the left anterior descending artery, the collateral capillary blood flow per unit weight of tissue is dependent upon the size of the ischemic region (Fig. 4). During clamping of the trunk of this artery which made some 20 to 30 g of myocardium ischemic, collateral blood flows in the range of 6 to 21 ml/min/100 g were obtained. Distal occlusion of the artery or occlusion of single side branches was in most animals associated with relatively higher values of CCBF per unit weight. Some animals, however, did not

show significant differences in CCBF a phenomenon which may reflect a less adequate supply of collateral vessels It is reasonable to assume that the latter group of animals would have also increased their relative flow values if still more distal occlusions had been made

Some particular attention was paid to the determinations of the relative size of the ischemic region as reflected by the zero time intercepts of the fast and slow components respectively It was assumed that experimental alterations of cardiovascular parameters (blood pressure heart rate blood viscosity) might influence not only the collateral blood flow but perhaps also the mass of myocardial tissue which was subjected to restricted blood flow during coronary artery occlusion. Even if the quotients obtained in experiments with alternative occlusions were clearly dependent upon the size of the myocardial regions supplied by the different vessels occluded (Fig 1) they varied considerably in double determinations (Fig 3) This is apparently due to difficulties in standardizing the Krypton injections in making an instantaneous occlusion and in marking this moment exactly on the curve Small or moderate changes in these quotients must evidently be judged with great caution

The rapid phase of the composite wash out curve obtained during coronary occlusion showed a rate constant which closely correlated with that of the monoexponential curve obtained in a preceding or following determination of normal myocardial blood flow i e when the coronary artery was left open It may be questioned whether the rapid phase of the composite curve gives a true picture of tissue blood flow in the myocardial regions supplied by the patent branches of the artery It has been reported that embolization of one arterial branch produces reflex changes of vascular tone in other parts of the coronary vasculature Studies of such intercoronary reflexes have given conflicting results Some authors report vasoconstriction others vasodilation (e g Guzman Swenson and Jones 1962 West *et al* 1962) Myocardial blood flow outside the region of ischemia was determined selectively in the experiment described in Table I The blood flow values found under these circumstances were not systematically different from the control values obtained when no coronary artery was clamped The results in Table I indicate also that the rapid phase of the composite curve is representative of blood flow outside the ischemic region The present study thus lends no support to the existence of intercoronary reflexes activated by arterial occlusion One possible reason for this negative result could be that the nervous connections mediating such reflexes had been interrupted by the dissection of the arteries

The present study was supported by grants from Svenska Nationalföreningen mot Hjärt och Lungsjukdomar

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From the Department of Anatomy University of Helsinki Finland

Fibres Containing both Noradrenaline and Acetylcholinesterase in the Nerve Net of the Rat Iris

By

OLAVI ERANKO and LIISA RAISANEN

Ganglion cells which contain both noradrenaline (NA) and acetylcholinesterase (AChE) have been demonstrated in the superior cervical ganglion of the rat (Eranko and Harkonen 1964) In the present report observations are presented showing that NA and AChE are present together also in some fine fibres of the sympathetic nerve net of the iris

The iris was spread fresh on a clean slide and was allowed to dry NA and AChE were then demonstrated in each preparation as described recently (Eranko 1964) The dry iris was first exposed to formaldehyde vapour to render NA fluorescent and photographed It was then subjected to Gomori s (1952) modification of Koelle s cholinesterase method and photographed again

As applied to iris the method proved difficult because formaldehyde tended to destroy all AChE activity in it However by reducing the exposure to formaldehyde just long enough to induce NA fluorescence about 20 min at 40 C AChE activity was still demonstrable thereafter

The distributions of NA and AChE were compared by superimposing the pairs of photomicrographs made of each iris preparation It was thus possible to compare the two reactions in the fine tortuous fibres more easily than in pictures examined side by side Fibres with NA appeared white against dark background in the fluorescence photograph and those with AChE activity appeared dark against light background in the other photograph Accordingly fibres which were white in one picture and dark in the other disappeared when accurately superimposed

Many of the finest fibres of the iris obviously belonging to the terminal sympathetic nerve net not only exhibited fluorescence due to NA but also showed AChE activity Other fibres were fluorescent but did not exhibit AChE activity and *vice versa* This suggests that the innervation apparatus of the iris includes 3 types of fibres *i e* fibres containing both NA and AChE and fibres containing either NA or AChE

Confirming our preliminary observations on the iris (Eranko and Harkonen 1964) the results now presented further strengthen the hypothesis of the existence of sympathetic fibres which are at the same time adrenergic and cholinergic (Burn and Rand 1962 Koelle 1962) They also complement Richardson s (1964) recent electron microscopic observations according to which some synapses of the iris presumably the cholinergic ones contain synaptic vesicles only while others presumably the adrenergic

ones contain both synaptic vesicles and electron-dense probably catecholamine containing droplets

However Richardson (1964) also showed that fine nerve fibres often run so close together that they probably appear as one fibre under the light microscope The demonstration in the present work of both NA and AChE apparently in single fibres may therefore be due to closely associated but separate fine fibres some of which contain NA and others which exhibit AChE activity In view of the existence of ganglion cells containing both NA and AChE in the superior cervical ganglion (Eränkö and Härkönen 1964) from which originate the sympathetic fibres of the iris the real existence of similar peripheral fibres appears more likely Even if adrenergic and cholinergic fibres of the iris should be separate they appear to be close enough to make possible the interaction of cholinergic and adrenergic mechanisms as proposed by Burn and Rand (1962)

During Professor G B Koelle's visit to our laboratory in August 1964 it became apparent that observations essentially similar to those reported in the present paper have independently been made in his laboratory in the University of Pennsylvania We gratefully acknowledge stimulating discussions with him

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From the Department of Pharmacology University of Lund Sweden

The Dynamics of the Inotropic Changes Produced by Ouabain and Increased Contraction Rate

By

K. A. P. Edman and E. Nilsson

The aim of the present investigation has been to explore the dynamic mechanisms of the inotropic changes produced by ouabain and increased contraction frequency in rabbit papillary muscles

The state of activity of the contractile unit (Hill 1949) at various moments after stimulation was determined by using the shortening velocity at a small load (50—100 mg) as an index according to the quick release method used previously in skeletal muscle (Jewell and Wilkie 1958) Almost the whole time course of the active state of the papillary muscle can be adequately determined in this way the degree of activity at the various moment being expressed as a fraction of the intensity at full activity The experimental procedure also made it possible to measure the series elastic compliance (see Wilkie 1956) The force velocity relation was determined by afterloaded contractions in close connection with the measurements of the time course of the activite state Simultaneous recordings of tension and shortening could be made by mounting the preparation between an isotonic lever (static friction 2 mg equivalent mass 115 mg photoelectric recording of movements) and an RCA 5734 transducer fitted in the bath

Fig 1 illustrates the force velocity relation of a papillary muscle (30 C) at contraction frequencies of 30 per min and 75 per min in ordinary Ringer's solution and at 30 contractions per min in the presence of 0 48 μM ouabain As can be seen ouabain and increased frequency affect the force velocity curve in a very similar way causing an almost parallel shift of the curve away from the origin The intersection of the force velocity curves with the abscissa represents the capacity of the contractile unit to produce isometric tension during full activity i e the maximal intensity of active state at the different situations studied Knowing the isometric twitch tension and the fraction of the maximal activity of the contractile unit at the moment when the peak tension is reached the actual value of the maximal capacity to produce tension can be derived The values so estimated plotted in Fig 1 fit well into the extrapolated parts of the force velocity curves It is concluded that ouabain and increased frequency both increase the maximal intensity of the active state

Fig 2 (A C) illustrates the *time course* of the active state of a rabbit papillary muscle under conditions similar to those in Fig 1 At a contraction rate of 30 per min in ordinary Ringer (A) full activity is reached at about 90 msec after stimulation and activity begins to decline at about 135 msec The activity has declined to about 80 of the plateau value at the moment when the isometric twitch is maximum Ouabain (C) increases the rate of rise of the activity leading to attainment of full intensity at an earlier moment after stimulation The decay of activity also starts earlier resulting in a marked reduction of the duration of the active state Similar effects are produced by increases in frequency (B However for a given increase in the maximal capacity

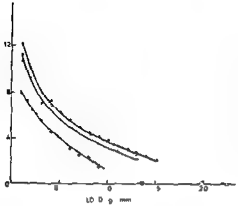

Fig 1 Force velocity curves of rabbit papillary muscle at contraction rate 30 per min (●) and 75 per min (○) in ordinary Ringer's solution and 120 min after introduction of 0.48 μM ouabain at frequency 30 per min (△) Temp 30° C Experimental data refer to different moments after stimulation depending of the load used Dashed lines after correction of experimental data by a factor determined by the ratio of the maximal intensity of active state to the degree of activity at the moment of recording Extrapolation indicated by dots For the estimation of loads corresponding to zero velocity see text

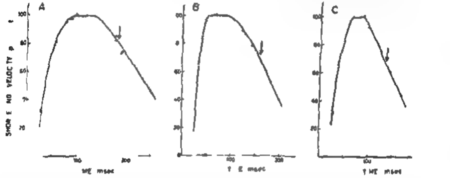

Fig 2 Active state curves of rabbit papillary muscle at contraction rate 30 per min (A) and 75 per min (B) in ordinary Ringer's solution and 140 min after introduction of 0.48 μM ouabain at frequency 30 per min (C) Ordinate velocity of shortening of contractile unit in per cent of velocity at full activity load 50 mg Abscissa time after stimulus Arrows indicate the time at which twitch tension is maximum

to produce tension the duration of activity is less affected by the frequency change than by ouabain — No substantial alteration of the series elastic compliance occurs in association with the inotropic effects studied

Conclusions

The potentiation of the isometric twitch amplitude as observed in papillary muscles produced by ouabain and increased frequency can be attributed both to an increase of the maximal intensity of the active state and to the fact that full activity of the contractile unit is attained earlier after stimulation of the cell The decrease in the duration of the active state is the primary cause of the reduction of the time to peak tension associated with the positive inotropic effects

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Catecholamines in Adrenals from Fetal Rabbits

By

Tomas Brundin

It has been generally assumed that noradrenaline is the only catecholamine present in adrenals from rabbit fetuses and that adrenaline does not appear until after birth (Shepherd and West 1951). However, this preliminary report presents evidence for the appearance of considerable quantities of adrenaline in rabbit adrenals already during their intrauterine development.

Albino rabbit fetuses were removed from the uterus at different times after conception. They were immediately decapitated and the adrenals were dissected out under a microscope. The catecholamines in the organs were estimated according to Euler and Lishajko (1961). Adrenaline was found in all organs analyzed (Fig. 1). The adrenaline quantities exceeded those of noradrenaline from the 20th day after conception and showed a large increase towards the end of gestation at about 30 days after conception. The relatively high adrenaline values fluorimetrically found were confirmed by bioassay on the hen's rectal caecum and by separation on an Amberlite column (Fig. 2).

Significantly lower adrenaline values were found in the fetal adrenals when the mother was previously anesthetized with urethane instead of being killed by a blow on the head before the fetuses were removed.

After fetal asphyxia a similar decrease of adrenaline content in the suprarenal gland was observed. This effect could be demonstrated as early as 20 days after conception. If this decrease is due to a true release of adrenaline, then a functioning adrenomedullary system exists already during fetal life. Work is in progress on these functions in rabbit and other mammalian fetuses.

This work has been supported by grants from Karolinska Institutet and from Stiftelse *Lars Hiertas Minne*.

Fig 1 Mean values of noradrenaline and adrenaline in fetal rabbit adrenals at different times after conception Vertical bars standard error of mean

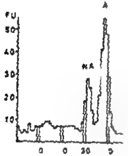

Fig Column separation of noradrenaline and adrenaline in adrenals from rabbit fetuses at full term (8 glands pooled) Ordinate fluorescence units Abscissa fraction numbers

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From the Department of Anatomy University of Helsinki Finland

Monoamine Containing Small Cells in the Superior Cervical Ganglion of the Rat and an Organ Composed of Them

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

Small cells have been observed in the superior cervical ganglion of the rat which exhibit an extremely bright yellow fluorescence after exposure to formaldehyde (Eränkö and Härkönen 1963 see also Fig 1 in Eränkö and Härkönen 1965) The present report describes some properties of these cells and an organ composed of them

Freeze dried ganglia were exposed to formaldehyde as described previously (Eränkö 1964) The fluorescence in the small cells was not only brighter and of different colour than that in the ganglion cells but it was also resistant to treatment with distilled water while breathing once on the section entirely abolished the green fluorescence of the ganglion cells After staining in 1 thionin which did not either destroy the fluorescence in the small cells (Fig 1) no colour was seen in the cytoplasm (Fig 2) This shows that the cells were not mast cells which is of interest because these also exhibit an intense yellow fluorescence resistant to water (unpublished observations see also Lagunoff et al 1961)

Complete series of sections were prepared from ganglia fixed in 3 5 % potassium dichromate No or few chromaffin cells were found in each ganglion This indicates that the far more numerous small cells must be non-chromaffin It is therefore somewhat surprising that cells of the same size and shape as the strongly fluorescent ones were electron microscopically observed to closely resemble the chromaffin cells of the adrenal medulla containing like these numerous intensely osmiophilic granules (Eränkö and Hanninen to be published)

It thus seems that the small cells contain high concentrations of a monoamine perhaps 5 hydroxytryptamine apparently stored in secretory granules in the same manner as catecholamines in the adrenal medulla They represent a new variety of non-chromaffin amine storing cells

While the small cells were scattered in the superior cervical ganglion especially near and in nerve bundles a well vascularized organ composed of these cells was accidentally observed near the ganglion (Fig 3) The organ was first thought to be the carotid body but this was subsequently found to be composed of cells exhibiting a green less intense fluorescence Therefore the small cells apparently form a hitherto unknown endocrine gland which perhaps secretes 5-hydroxytryptamine

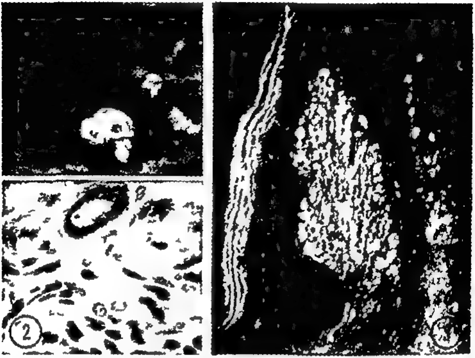

Fig 1 Fluorescence photomicrograph of a freeze-dried ganglion exposed to formaldehyde and stained with thionin Two small cells still fluoresce intensely 600 ×

Fig 2 Same field as in Fig 1 photographed in transmitted visible light Ganglion cells and nuclei are stained but the cytoplasm of the two small cells is unstained 600

Fig 3 Organ composed of the small intensely fluorescent cells in the centre On the left wall of an artery On the right the superior cervical ganglion Formaldehyde induced fluorescence 75

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